IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	LOSKUTOFF ET AL.	Examiner:	V. AFREMOVA
Serial No.:	10/044,796	Group Art Unit:	1657
Filed:	JANUARY 11, 2002	Docket No.:	13511.1USU1
Title:	SEMEN EXTENDER COMPOSITION AND METHODS FOR MANUFACTURING AND USING		

CERTIFICATE UNDER 37 CFR 1.8:
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By: Mona Steil
Name:

APPELLANT'S BRIEF ON APPEAL

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23552
PATENT TRADEMARK OFFICE

Sir:

This Brief is presented in support of the Notice of Appeal filed on July 16, 2008, from final rejection of Claims 1, 2, 4, 5, 9, 14, 21, 24-26, 28-31, and 33 of the above-identified application, as set forth in the Office Action mailed January 16, 2008.

Please charge Deposit Account No. 13-2725 $270.00 to cover the required fee for filing this Brief.

An oral hearing is requested. A separate request for oral hearing with the appropriate fee will be filed within two months of the Examiner's Answer.

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I. REAL PARTY OF INTEREST

The assignee of the above-identified patent application is ABS Corporation. ABS Corporation is the real party in interest.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals and interferences.

III. STATUS OF CLAIMS

Claim 1, 2, 4, 5, 9, 14, 21, 24-26, 28-31, and 33 are pending in this application, and are rejected. Claims 1, 2, 4, 5, 9, 14, 21, 24-26, 28-31, and 33 are on appeal.

IV. STATUS OF AMENDMENTS

No amendment to the claims has been submitted or introduced subsequent to the Office Action mailed on January 16, 2008. All amendments mailed prior to the mailing date of the Office Action mailed on January 16, 2008 have been entered.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The invention according to independent claim 1 is directed to an aqueous ready to use semen extender composition. The composition includes: about 0.1 wt.% to about 6 wt.% phospholipid obtained from a non-animal source comprising lecithin (page 5, lines 3-7 and 13-19); about 0.0001 wt.% to about 1 wt.% of anionic surfactant to reduce ice crystal formation during freezing of the composition (page 6, lines 4-6 and 26-29), wherein the anionic surfactant comprises a sulfate (page 6, lines 12-13); about 0.5 wt.% to about 3 wt.% carbohydrate (page 5, line 24 through page 6, line 1); about 3 wt.% to about 14 wt.% freeze agent comprising glycerol (page 7, lines 16-21); and biological buffer to provide the composition with a pH of about 6.9 to about 7.5 (page 7, lines 1-3), and wherein the composition comprises a sufficient amount of water so that the composition exhibits an osmolality of about 250 mOsM to about 350 mOsM (page 4, lines 19-29), wherein the composition is substantially free of animal products (page 3, lines 8-22). See page 1, line 24 through page 2, line 2.

The invention according to independent claim 21 is directed to a method for manufacturing an aqueous ready to use semen extender composition. The method comprises mixing semen extender composition components to provide a semen extender composition having an osmolality of about 250 mOsM to about 350 mOsM and a pH of about 6.9 to about 7.5 (page 4, lines 19-29, and page 7, line 1-3). The semen extender composition components comprising: about 0.1 wt.% to about 6 wt.% phospholipid obtained from a non-animal source comprising lecithin (page 5, lines 3-7 and 13-19); about 0.0001 wt.% to about 1 wt.% of anionic surfactant to reduce ice crystal formation during freezing of the composition (page 6, lines 4-6 and 26-29), wherein the anionic surfactant comprises a sulfate (page 6, lines 12-13); about 0.5 wt.% to about 3 wt. carbohydrate (page 5, line 24 through page 6, line 1); about 3 wt.% to about

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

There are two grounds of rejection to review on Appeal. Both grounds of rejection are identified below.

I. Claims 1, 2, 4, 5, 9, 14, 21, 24-26, 28-31, and 33 stand rejected under 35 U.S.C. § 112, first paragraph.

II. Claims 1, 2, 4, 5, 9, 14, 21, 24-26, 28-31, and 33 stand rejected under 35 U.S.C. § 103(a) over *Rota et al.* ("Fertility After Vaginal Or Uterine Deposition Of Semen Frozen In A Tris Extender With Or Without STM Paste," Theriogenology, (April 15, 1999) Vol. 51, No. 6, pages 1045-1058), European Patent No. EP 0 685 556 to *Ghazarian et al.*, U.S. Patent No. 3,444,039 to *Rajamannan*, U.S. Patent No. 6,130,034 to *Aitken*, U.S. Patent No. 6,140,121 to *Ellington et al.*, C. Hellemann, and E. Giegoux, Deep Freezing of Rabbit Sperm, Effect of a Surfactant on Fertilizing Capacity, Zuchthyg., 23, 33-37 (1988)(*Hellemann et al.*).

14 wt.% freeze agent comprising glycerol (page 7, lines 16-21); water (page 4, lines 19-29); and biological buffer(page 7, lines 1-3), wherein the composition is substantially free of animal products (page 3, lines 8-22). See page 2, lines 12-17.

Both the inventive aqueous ready to use semen extender composition according to independent claim 1 and the inventive method according to independent claim 21 avoid the use of raw egg yolk which is a common component of prior art semen extender compositions while retaining a desired level of performance. Animal products, such as raw egg yolk, may contain nonpathogenic organisms and pathogenic organisms harmful to the host or cells provided in contact with the egg yolk. See page 3, lines 23-28.

VII. ARGUMENT

Rejection Under 35 U.S.C. § 112, First Paragraph

The outstanding Office Action includes a rejection under 35 U.S.C. § 112, first paragraph. Reversal of this rejection is requested.

The outstanding Office Action states:

> "Insertion of the limitation directed to the use of the entire concentration range 0.0001 % to 1 % of sulfate-containing anionic surfactant in the semen extender composition with lecithin has no support in the as-filed specification. The insertion of this limitation is a new concept because it neither has literal support in the as-filed specification by way of generic disclosure, nor are there specific examples of the newly limited genus that would show possession of the concept of the use of the entire concentration range 0.0001% to 1% of sulfate-containing anionic surfactant."

See the outstanding Office Action at pages 2-3.

The specification at page 6, lines 4-31, clearly supports the incorporation of an anionic surfactant in a range of about 0.0001 wt.% to about 1 wt.% and wherein the anionic surfactant comprises a sulfate. In particular, the specification at page 6, lines 4-6, states: "[t]he semen extender composition can include a surfactant to help reduce ice crystal formation during freezing process and to help strengthen the cell membrane during the freezing and thawing process." The specification at page 6, lines 10-13, identifies anionic surfactants as a type of surfactant that can be used, and specifically identifies sulfates as a type of anionic surfactant that can be use. In addition, the specification at page 6, lines 12-13, identifies sodium lauryl sulfate and sodium laureth sulfate as specific sulfate surfactants that can be used. Furthermore, the specification at page 6, lines 20-31, provides that the semen extender composition can include a single surfactant or a mixture of surfactants, and identifies a range of about 0.0001 wt.% to about 1 wt.% surfactant. Furthermore, the specification at page 12, line 17, specifically offers a range of sodium lauryl sulfate that can be used in an example as 0.01 wt.% to 1 wt.%. Clearly, the range identified in the specification at page 6, lines 20-31, is a generic range applicable to all the surfactants identified in the specification at page 6, lines 4-19, and the range disclosed in the

specification at page 12, line 17, is an exemplary range for a specific component (e.g., sodium lauryl sulfate which is an example of an anionic surfactant).

The outstanding Office Action states:

> "The generic disclosure of the as-filed specification (page 6) describes a variety of surfactants including anionic, cationic, nonionic, etc. (page 6) with nonionic surfactant including glycerol esters and Tween 80 as preferred for the semen extender compositions."

See the outstanding Office Action at page 3. This statement in the outstanding Office Action is not entirely correct. The specification at page 6, lines 4-19, describes surfactants that can be used. In general, the surfactants that can be used are those surfactants that are characterized as sufficiently gentle so that they do not dissolve sperm cell membrane. Anionic surfactants such as sulfates (and in particular sodium lauryl sulfate and sodium laureth sulfate) are identified as exemplary surfactants. The specification further identifies sorbitan ester (a nonionic surfactant) as an exemplary surfactant. An exemplary sorbitan ester is stated to include polyoxyethylene sorbitan monooleate. The specification at page 6, lines 16-19, states that a "preferred polyoxyethylene sorbitan monooleate is available under the name Tween 80 from Sigma Chemical Company." This is not a statement that Tween 80 is the preferred surfactant or that nonionic surfactants are the preferred surfactants. Instead, this is simply a statement that Tween 80 is a preferred polyoxyethylene sorbitan monooleate. The statement in the outstanding Office Action that glycerol esters and Tween 80 are preferred for semen extender compositions is not a statement that can be reasonably inferred from a correct grammatical reading of the specification at page 6, lines 4-19.

The outstanding Office Action states that "the claimed concentration range 0.0001% to 1% is linked to the use of nonionic surfactant as disclosed (page 6, lines 25-31)." See page 3 of the outstanding Office Action. This contention made in the outstanding Office Action is not correct. Clearly, the specification describes many types of surfactants at page 6, lines 4-19. In fact, sulfates are identified as a type of anionic surfactant. See the specification at page 6, lines 12-13. The range "between about 0.0001 wt.% and about 1 wt.% surfactant" provided in the specification at page 6, lines 27-28, clearly is a generic reference to the surfactants identified in the specification at page 6, lines 4-19. The subsequent range of "between 0.001 wt.% and about

0.005 wt.%" is clearly an exemplary range for polyoxyethylene sorbitan monooleate. Thus, the interpretation in the outstanding Office Action that the range of 0.0001 wt.% to 1 wt.% surfactant is limited only to nonionic surfactants is clearly erroneous.

The contention that the range of sulfate surfactant in the claims is not supported by the specification is erroneous, and reversal of the rejection is requested.

Rejection Under 35 U.S.C. § 103(a)

Claims 1, 2, 4, 5, 9, 11, 14, 21, 22, 24-26, 28-31, and 33 stand rejected under 35 U.S.C. § 103(a) over Rota et al.("Fertility After Vaginal Or Uterine Deposition Of Semen Frozen In A Tris Extender With Or Without STM Paste," Theriogenology, (April 15, 1999) Vol. 51, No. 6, pages 1045-1058), European Patent No. EP 0 685 556 to *Ghazarian et al.*, U.S. Patent No. 3,444,039 to *Rajamannan*, U.S. Patent No. 6,130,034 to *Aitken*, U.S. Patent No. 6,140,121 to *Ellington et al.*, C. Hellemann, and E. Giegoux, Deep Freezing of Rabbit Sperm, Effect of a Surfactant on Fertilizing Capacity, Zuchthyg., 23, 33-37 (1988)(*Hellemann et al.*) Reversal of this rejection is requested.

The invention according to independent claim 1 is directed to an aqueous ready to use semen extender composition. The composition includes: about 0.1 wt.% to about 6 wt.% phospholipid obtained from a non-animal source comprising lecithin; about 0.0001 wt.% to about 1 wt.% of anionic surfactant to reduce ice crystal formation during freezing of the composition, wherein the anionic surfactant comprises a sulfate; about 0.5 wt.% to about 3 wt.% carbohydrate; about 3 wt.% to about 14 wt.% freeze agent comprising glycerol; and biological buffer to provide the composition with a pH of about 6.9 to about 7.5, and wherein the composition comprises a sufficient amount of water so that the composition exhibits an osmolality of about 250 mOsM to about 350 mOsM, wherein the composition is substantially free of animal products. The invention according to independent claim 21 is directed to a method for manufacturing an aqueous ready to use semen extender composition. The method comprises mixing semen extender composition components to provide a semen extender composition having an osmolality of about 250 mOsM to about 350 mOsM and a pH of about 6.9 to about 7.5. The resulting semen extender composition includes the features provided by independent claim 1.

The semen extender composition according to the invention provides for the substantial absence of animal products. For example, many prior art semen extender compositions use raw egg yolk as a necessary component. Raw egg yolk is excluded from the semen extender composition according to the invention. Animal product, such as, egg yolk, may contain nonpathogenic or pathogenic organisms harmful to the host or cell provided in contact with the animal product. While the Applicants are not the first to provide a semen extender composition free of animal products, they have provided a semen extender composition that can perform effectively compared with egg yolk-based semen extender compositions without worry or risk about the existence of potential harmful organisms that may be present with animal based products such as egg yolk.

Rota et al. is relied upon for the disclosure of "a semen extender composition with 0.5% of an anionic sulfate-containing such as sodium lauryl sulfate (Equex STM paste) and 5% of glycerol in a regular egg yolk-tris-citrate extender (abstract)." See the outstanding Office Action at page 5. Rota et al., however, teach the use of an egg yolk semen extender compositon wherein the present invention is substantially free of animal products such as egg yolk. Furthermore, *Rota et al.* teach that sodium lauryl sulfate is effective with egg yolk. There is no teaching or suggestion by *Rota et al.* to use sodium lauryl sulfate in the absence of egg yolk. In fact, one having ordinary skill in the art would recognize that *Rota et al.* teach the necessary combination of sodium lauryl sulfate and egg yolk to achieve a desired result. According to *Rota et al.*:

> "In a previous study, we found that the addition of Equex STM Paste to a freezing extender improved the longevity in vitro of frozen-thawed dog spermatiozoa (35). Equex STM Paste is a commercially available additive for use with semen extenders. <u>It contains sodium dedecyl sulphate (SDS), a detergent thought to act through an alteration of the egg yolk</u>, possibly increasing its protecting potential against cold shold and freezing injury (31)."

Rota et al. at page 1046 (emphasis added). In the quotation by *Rota et al.* at page 1046, reference is made to a previous study reported as reference 35. This previous study is *Rota et al.*, "Effects Of Equex STM Paste On Viability Of Frozen-Thawed Dog

Spermatozoa During In Vitro Incubation At 38 C," Theriogenology 1997; 47:1093-1101 (hereinafter *Rota et al.'1997*). *Rota et al.'1997* compare vaginal deposition and uterine deposition for artificial insemination of canines with cryopreserved semen. The results of the tests are discussed by *Rota et al.'1997* on page 1098. According to *Rota et al.'1997*:

> "The present results clearly demonstrate that the addition of Equex STM Paste is beneficial for freezing dog semen. Both viability and longevity post-thaw were enhanced, as had been observed in the findings of Thomas et al. (1992). The active compound in Equex STM Paste is thought to be the detergent SDS, which probably exerts its action through the alteration of the egg yolk contained in the extender (22). The interaction with the egg yolk could involve the solubilization of active molecules. This hypothesis is supported by the observation that when only the supernatant was used in preparing the extenders, SDS had a stronger positive effect on post-thaw motility when added to the egg yolk before centrifugation than when added later (21). Although a direct positive effect of SDS on the plasma membrane has also been hypothezised, Pursel et al. (22) found reduced sperm viability when SDS was added to extender depleted of egg yolk, thus indicating the contrary. Moreover, detergents by themselves, including SDS, are known to have spermicidal effect (17)."

Rota et al.'1997 at page 1098 (emphasis added).

Both *Rota et al.* and *Rota et al.'1997* report an interaction between sodium lauryl sulfate and egg yolk. That observation clearly indicates that both *Rota et al.* and *Rota et al.'1997* teach the presence of egg yolk when using Equex STM Paste (sodium layuryl sulfate). Furthermore, *Rota et al.'1997* report another teaching that reduced sperm viability was observed "when SDS [sodium lauryl sulfate] was added to extender depleted of egg yolk." See *Rota et al.'1997* at page 1098. Furthermore, this location additionally recognizes that detergents such as sodium lauryl sulfate "are known to have spermicidal effect." See *Rota et al.'1997* at page 1098. Clearly, this is teaching away from the use of sodium lauryl sulfate in a semen extender composition when no egg yolk is present.

It is submitted that the recent Supreme Court Decision *KSR International Co. v. Teleflex Inc.*, 127 S.Ct. 1727 (2007), does not alter the established law relating to "teaching away." For example, "[p]roceeding contrary to accepted wisdom in the art is evidence of nonobviousness." *In re Hedges*, 783 F.2d 1038 (Fed. Cir. 1986). In the present situation, both *Rota et al.* and *Rota et al.'1997* teach the required combination of sodium lauryl sulfate and egg yolk, and teach away from using sodium lauryl sulfate in the absence of egg yolk. Accordingly, both *Rota et al.* and *Rota et al.'1997* teach away from the present invention.

The outstanding Office Action relies upon Ghazarian et al. for the disclosure of a semen extender composition that is soy lecithin based rather than egg yolk based. *Ghazarian et al.*, however, fail to disclose an aqueous ready to use semen extender composition comprising about 0.0001 wt.% to about 1 wt.% of anionic surfactant comprising a sulfate to reduce ice crystal formation during freezing of the composition according to the present invention. As discussed above, *Rota et al.* and *Rota et al.' 1997* are directed at egg yolk based compositions, and teach away from the use of sodium lauryl sulfate when a composition does not contain egg yolk.

Ghazarian et al. are directed to a vehicle for nonautonomous microorganisms of the animal kingdom to be kept alive outside their natural environment with a view to human interventions. The vehicle includes an aqueous medium comprising nutrition agents, buffers and mineral salts, and a protective product formed as a support for embryonic growth by a living organism, wherein the protective product is a lecithin extracted from soy seeds and introduced into the aqueous medium upon formation of the vehicle. See the English language translation of *Ghazarian et al.* on page 2, lines 1-17, and page 3, lines 20-27.

As discussed above, *Ghazarian et al.* fail to disclose a composition containing about 0.0001 wt.% to about 1 wt.% of anionic surfactant, comprising a sulfate, to reduce ice crystal formation during freezing of the composition.

Rajamannan, *Aitken*, *Ellington et al.*, and *Hellemann et al.* would not have suggested modifying *Rota et al.* or *Ghazarian et al.* to include about 0.0001 wt.% to about 1 wt.% of anionic surfactant comprising a sulfate to reduce ice crystal formation during freezing of the composition according to the presently claimed invention.

Rajamannan appears to be relied upon in the outstanding Office Action for the disclosure of buffering to a pH of 6 to 7.5 and for the disclosure of sodium citrate as a buffering agent. See

Rajamannan at column 3, line 30 and lines 41-47. It is pointed out that *Rajamannan* is directed at an egg yolk containing composition. See *Rajamannan* at column 1, lines 13-19, and the example disclosing the presence of egg yolk solids. Accordingly, *Rajamannan* is representative of prior art compositions that are based on the use of egg yolk. In contrast, the present invention is an improvement over prior art egg yolk-based compositions. Furthermore, *Rajamannan* fails to disclose or suggest the use of about 0.0001 wt.% to about 1 wt.% anionic surfactant comprising a sulfate to reduce ice crystal formation during freezing of the composition according to the present invention.

It appears that the outstanding Office Action relies upon *Aitken* for the disclosure of an anti-oxidant. *Aitken* refers to an anti-oxidant such as vitamin E at column 1, line 50. It is pointed out, however, that Aitken is also directed at an egg yolk-containing system. See *Aitken* at column 1, lines 28-38. Clearly, *Aitken* is similarly representative of a prior art composition containing egg yolk. In contrast, the present invention is directed at a semen extender composition that is substantially free of animal products such as egg yolk. As discussed in the specification of the above-identified patent application beginning at page 3, line 23, it is believed that animal products, such as egg yolk, may contain nonpathogenic organisms or pathogenic organisms harmful to the host or cell provided in contact with the animal product. Accordingly, the present invention is directed at an improvement over those compositions that contain animal product. The outstanding Office Action fails to explain why one having ordinary skill in the art would look to a disclosure relating to the use of raw egg yolk for a suggestion to modify a composition that is free of raw egg yolk.

It is submitted that raw egg yolk contains a large number of various components and is a much more complicated system than the semen extender composition that does not contain raw egg yolk according to the present invention. Accordingly, the disclosure of the use of an anti-oxidant in a raw egg containing semen extender composition according to *Aitken* in no way suggests the use of an anti-oxidant in a non-raw egg containing semen extender composition.

Nevertheless, the outstanding Office Action fails to explain why one having ordinary skill in the art would have received a suggestion from *Aitken* to modify *Ghazarian et al.* to include about 0.0001 wt.% to about 1 wt.% anionic surfactant comprising a sulfate to reduce ice crystal formation during freezing of the composition according to the present invention.

The outstanding Office Action appears to rely on *Ellington et al.* for the disclosure of various buffers such as EDTA and TRIS. See *Ellington et al.* at column 16, lines 52-63, and column 19, line 28. The outstanding Office Action additionally refers to *Ellington et al.* for the disclosure of a balanced culture medium such as M199 at column 16, line 59, and contends that medium M199 suggests the use of polyoxyethylene sorbitan (Tween 80). It is submitted that Tween 80 is provided in medium M199 to help dissolve the other components in medium M199. There is no disclosure by *Ellington et al.* or ATCC Catalogue (Page 522) that Tween 80 can be useful for reducing ice crystal formation during freezing of a semen extender composition. One having ordinary skill in the art would not have received any suggestion from *Ellington et al.* or ATCC Catalogue (Page 522) that the incorporation of Tween 80 into the composition described by *Ghazarian et al.* would have any benefit for reducing ice crystal formation during freezing according to the present invention.

The reliance upon *Ellington et al.* and ATCC Catalogue (Page 522) is an example of the use of impermissible hindsight. There must be a suggestion to combine the references or make the modifications to achieve a *prima facie* case of obviousness. It is not enough to simply pick and choose various components from several references. The outstanding Office Action fails to explain why one having ordinary skill in the art would be motivated to select Tween 80 from the lengthy list of components identified in balanced culture M199, and then add that component to the composition described by *Ghazarian et al.* Nevertheless, it is pointed out that Tween 80 is an example of a nonionic surfactant. There is no suggestion by *Ellington et al.* that an anionic surfactant comprising a sulfate can be used to reduce ice crystal formation during freezing according to the present invention.

Hellemann et al. are apparently relied upon in the outstanding Office Action for the disclosure of sodium laurel sulfate in a composition intended for rabbit semen. See the abstract of *Hellemann et al.* Similar to *Rajamannan* and *Aitken*, *Hellemann et al.* are directed at compositions containing raw egg. Accordingly, *Hellemann et al.* are yet another example of a representative prior art composition that relies upon the use of raw egg yolk. In contrast, the present invention is directed at an improvement over those compositions that are based upon animal products such as raw egg yolk. The Examiner's attention is directed to the specification at, for example, page 3, line 8 through page 4, line 2. It is submitted that one having ordinary

skill in the art would not have looked to *Hellemann et al.* for modifying a composition that does not contain raw egg yolk. Furthermore, the outstanding Office Action fails to provide a sufficient reason to explain why one having ordinary skill in the art would modify *Ghazarian et al.* in view of the disclosure by *Hellemann et al.* to achieve the presently claimed invention.

The outstanding Office Action on page 6, merely states "the reference by Hellemann et al. is relied upon for the teaching about the use of sodium lauryl sulfate in the composition intended for animal semen preservation (see English abstract)." However, *Hellemann et al.* fail to explain why one having ordinary skill in the art would be motivated to include sodium lauryl sulfate in a composition for freezing rabbit semen. Furthermore, one having ordinary skill in the art would not, from reading *Hellemann et al.*, understand why sodium lauryl sulfate would be included in a raw egg yolk-base composition, and would certainly not receive a suggest to include sodium lauryl sulfate in a semen extender composition that is substantially free of animal products. *Hellemann et al.* clearly provide no suggestion on how to modify a composition that is substantially free of raw egg yolk.

Finally, *Hellemann et al.* teach the use of an anionic surfactant to protect against damage caused by dimethyl sulfoxide (DMSO). See the first full paragraph of the translation of *Hellemann et al.* Accordingly, *Hellemann et al.* are concerned with the affect of sodium lauryl sulfate on a composition that utilizes DMSO. The results reported by *Hellemann et al.* are somewhat ambiguous but it appears that *Hellemann et al.* teach away from the use of sodium lauryl sulfate because it is detrimental except at high levels of DMSO where it is effective for increasing acrosomal activity. Accordingly, the general teachings of *Hellemann et al.* are to not use sodium lauryl sulfate unless there is a large presence of DMSO.

In contrast to *Hellemann et al.*, the present invention provides for the presence of a freeze agent comprising glycerol. The presently claimed invention does not require the presence of DMSO. In fact, because the claimed invention provides for the presence of glycerol, one would not include DMSO.

Clearly, one having ordinary skill in the art would not have received a suggestion from *Hellemann et al.* to modify *Rota et al.* or *Ghazarian et al.* to include sodium lauryl sulfate. *Hellemann et al.* teach the use of sodium lauryl sulfate in response to a composition containing a relatively high level of DMSO in order to increase acrosomal integrity. Because the *Ghazarian*

et al. do not include DMSO in their composition, it is submitted that one having ordinary skill in the art would not have received the suggestion from *Hellemann et al.* to modify *Ghazarian et al.* to include sodium lauryl sulfate.

In view of the comments, the presently claimed invention would not have been obvious from *Rota et al.*, *Ghazarian et al.*, *Rajamannan*, *Aitken*, *Ellington et al.*, and *Hellemann et al.* Accordingly, reversal of the rejection is requested.

SUMMARY

Reversal of all of the rejections and allowance of the pending claims are requested.

Please charge any additional fees or credit any overpayment to Merchant & Gould P.C., Deposit Account No. 13-2725

Respectfully submitted,

MERCHANT & GOULD P.C.
P.O. Box 2903
Minneapolis, Minnesota 55402-0903
(612) 332-5300

Date: November 17, 2008

Dennis R. Daley
Reg. No. 34,994
DRD:mls

CLAIMS APPENDIX

1. (Previously Presented) An aqueous ready to use semen extender composition comprising:

(a) about 0.1 wt.% to about 6 wt.% phospholipid obtained from a non-animal source comprising lecithin;

(b) about 0.0001 wt.% to about 1 wt.% of anionic surfactant to reduce ice crystal formation during freezing of the composition, wherein the anionic surfactant comprises a sulfate;

(c) about 0.5 wt.% to about 3 wt.% carbohydrate;

(d) about 3 wt.% to about 14 wt.% freeze agent comprising glycerol; and

(e) biological buffer to provide the composition with a pH of about 6.9 to about 7.5, and wherein the composition comprises a sufficient amount of water so that the composition exhibits an osmolality of about 250 mOsM to about 350 mOsM, wherein the composition is substantially free of animal products.

2. (Previously Presented) An aqueous ready to use semen extender composition according to claim 1, wherein the composition comprises at least about 90 wt.% water.

3. (Canceled)

4. (Previously Presented) An aqueous ready to use semen extender composition according to claim 1, further comprising:

(a) antioxidant.

5. (Previously Presented) An aqueous ready to use semen extender composition according to claim 4, wherein the antioxidant comprises at least one of vitamin E, vitamin C, vitamin A, BHA, BHT, or derivatives thereof.

6. (Canceled)

7-8. (Canceled)

9. (Previously Presented) An aqueous ready to use semen extender composition according to claim 1, wherein the anionic surfactant comprises at least one of sodium lauryl sulfate, sodium laureth sulfate, or mixtures thereof.

10. (Canceled)

11. (Canceled)

12. (Canceled)

13. (Canceled)

14. (Previously Presented) An aqueous ready to use semen extender composition according to claim 1, further comprising semen.

15-20. (Canceled)

21. (Presently Presented) A method for manufacturing an aqueous ready to use semen extender composition, the method comprising a step of:

(a) mixing semen extender composition components to provide a semen extender composition having an osmolality of about 250 mOsM to about 350 mOsM and a pH of about 6.9 to about 7.5, the semen extender composition components comprising:

(i) about 0.1 wt.% to about 6 wt.% phospholipid obtained from a non-animal source comprising lecithin;

(ii) about 0.0001 wt.% to about 1 wt.% of anionic surfactant to reduce ice crystal formation during freezing of the composition, wherein the anionic surfactant comprises a sulfate;

(iii) about 0.5 wt.% to about 3 wt.;

(iv) about 3 wt.% to about 14 wt.% freeze agent comprising glycerol;

(v) water; and

(vi) biological buffer, wherein the composition is substantially free of animal products.

22. (Canceled)

23. (Canceled)

24. (Previously Presented) An aqueous ready to use semen extender composition according to claim 1, wherein the composition comprises at least about 1 IU/ml antioxidant.

25. (Previously Presented) An aqueous ready to use semen extender composition according to claim 1, wherein the composition comprises at least about 5 IU/ml antioxidant.

26. (Previously Presented) An aqueous ready to use semen extender composition according to claim 1, wherein the composition comprises about 1 wt.% to about 3 wt.% antioxidant.

27. (Canceled)

28. (Previously Presented) A method according to claim 21, wherein the composition comprises at least about 1 IU/ml antioxidant.

29. (Previously Presented) A method according to claim 21, wherein the composition comprises at least about 5 IU/ml antioxidant.

30. (Previously Presented) A method according to claim 21, wherein the composition comprises about 1 wt.% to about 3 wt.% antioxidant.

31. (Previously Presented) A method according to claim 28, wherein the antioxidant comprises at least one of vitamin E, vitamin C, vitamin A, BHA, BHT, or derivatives thereof.

32. (Canceled)

33. (Previously Presented) A method according to claim 21, wherein the anionic surfactant comprises at least one of sodium lauryl sulfate, sodium laureth sulfate, or mixtures thereof.

34. (Canceled)

EVIDENCE APPENDIX

A. OFFICE ACTIONS AND AMENDMENTS/RESPONSES

1. Office Action -- mailed January 16, 2008

B. REFERENCES RELIED UPON BY THE EXAMINER

1. *Rota et al.* ("Fertility After Vaginal Or Uterine Deposition Of Semen Frozen In A Tris Extender With Or Without STM Paste," Theriogenology, (April 15, 1999) Vol. 51, No. 6, pages 1045-1058),

2. European Patent No. EP 0 685 556 to *Ghazarian et al.* (English language translation),

3. U.S. Patent No. 3,444,039 to *Rajamannan*,

4. U.S. Patent No. 6,130,034 to *Aitken*,

5. U.S. Patent No. 6,140,121 to *Ellington et al.*,

6. C. Hellemann, and E. Giegoux, Deep Freezing of Rabbit Sperm, Effect of a Surfactant on Fertilizing Capacity, Zuchthyg., 23, 33-37 (1988) (English language translation)

C. REFERENCES CITED BY APPELLANTS

1. *Rota et al.*, "Effects Of Equex STM Paste On Viability Of Frozen-Thawed Dog Spermatozoa During In Vitro Incubation At 38 C," Theriogenology 1997; 47:1093-1101 (hereinafter *Rota et al.'1997*).

D. CASES CITED IN THE BRIEF

1. *KSR International Co. v. Teleflex Inc.*, 127 S.Ct. 1727 (2007),

2. *In re Hedges*, 783 F.2d 1038 (Fed. Cir. 1986).

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10/044,796	01/11/2002	Naida M. Loskutoff	13511.1USU1	8344

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MERCHANT & GOULD PC
P.O. BOX 2903
MINNEAPOLIS, MN 55402-0903

DRD

RESP 3 MNH/ PTA: April 16, 2008
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EXAMINER	
AFREMOVA, VERA	
ART UNIT	**PAPER NUMBER**
1657	
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Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/044,796	LOSKUTOFF ET AL.	
	Examiner	Art Unit	
	Vera Afremova	1657	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *23 October 2007*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,2,4,5,9,14,21,24-26,28-31 and 33* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1,2,4,5,9,14,21,24-26,28-31 and 33* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Continued Examination Under 37 CFR 1.114

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 10/23/2007 has been entered.

Claims 1, 2, 4, 5, 9, 14, 21, 24-26, 28-31 and 33 as amended (10/23/2007) are pending and under examination.

Claim Rejections - 35 USC § 112

New matter

Claims 1, 2, 4, 5, 9, 14, 21, 24-26, 28-31 and 33 as amended remain/are rejected under 35 U.S.C. 112, *first paragraph*, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

Insertion of the limitation directed to the use of the entire concentration range 0.0001 % to 1 % of the sulfate-containing anionic surfactant in the semen extender composition with lecithin has no support in the as-filed specification. The insertion of this limitation is a new concept because it neither has literal support in the as-filed specification by way of generic disclosure, nor are there specific examples of the newly limited genus that would show

possession of the concept of the use of the entire concentration range 0.0001 % to 1 % of the sulfate-containing anionic surfactant.

The generic disclosure of the as-filed specification (page 6) describes a variety of surfactant including anionic, cationic, nonionic, etc. (page 6) with nonionic surfactant including glycerol esters and tween 80 as preferred for the semen extender compositions. The generic disclosure of the as-filed specification does not describe amounts as intended for an anionic surfactant separately. Moreover, the presently claimed concentration range 0.0001 % to 1 % is linked to the use of nonionic surfactant as disclosed (page 6, line 25-31) or at the very best to the mixture of various types of surfactants. The generic disclosure of the as-filed specification does not describe amounts as intended for a generic sulfate-containing anionic surfactant separately. As related to the presently claimed sulfate-containing anionic surfactant there is only one exemplified disclosure such as the use of sodium lauryl sulfate in amounts 0.01% - 1% (page 12, line 17).

This is not sufficient support for the entire range as presently claimed. This is a matter of written description, not a question of what one of skill in the art would or would not have known. The material within the four corners of the as-filed specification must lead to the generic concept. If it does not, the material is new matter. Declarations and new references cannot demonstrate the possession of a concept after the fact. Thus, the insertion of directed to the use of the entire concentration range 0.001 % to 1 % of sulfate containing anionic surfactant in the semen extender composition with lecithin is considered to be the insertion of new matter for the above reasons.

Please see *Gentry Gallery v. Berkline* 45 U.S.P.Q.2d 1498 for a discussion related to broadening the claimed invention without support in the as-filed specification.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 1, 2, 4, 5, 9, 4, 21, 24-26, 28-31 and 33 as amended are rejected under 35 U.S.C. 103(a) as being unpatentable over Rota et al. ("Fertility after vaginal or uterine deposition of doj semen frozen in a tris extender with or without Equex STM Paste". Theriogenology, (April 15, 1999) Vol.51, No. 6, pages 1045-1058), EP 0 685 556 (Ghazarian), US 3,444,039 (Rajamannan), US 6,130,034 (Aitken), US 6,140,121 (Ellington et al) and the reference by Hellmann et al. (Zuchthg., 1988, 23: 33-37).

Claims are directed to a semen extender composition that comprises a non-animal derived phospholipids such as lecithin in amounts of about 0.1-6%, about 0.0001-1% of anionic sulfate-containing surfactant, about 0.5-3% of carbohydrate, about 3-14% of freeze-agent comprising glycerol and a buffer to provide for pH of about 6.9-7.2 and osmolarity of about 250-350 mOsm. Some claims are further drawn to the use of 90% water in the composition. Some claims are further drawn to the use of anionic sulfate-containing surfactant comprising sodium lauryl sulfate. Some claims are further drawn to the use of antioxidants such as vitamin A or vitamin E

in the semen extender composition. Some claims are further drawn to incorporation of semen into the semen extender composition. Some claims are/are further drawn to the method of making the semen extender composition by mixing the components of the composition. Some claims are further drawn to the use of to the use of specific concentrations of anti-oxidant(s) in the semen extender composition.

The reference by Rota et al. discloses a semen extender composition with 0.5% of an anionic sulfate-containing such as sodium lauryl sulfate (Equex STM paste) and 5% of glycerol in a regular egg yolk-tris-glucose-citrate extender (abstract). The egg yolk-tris-glucose-citrate extender provides for egg yolk lecithin, carbohydrates, pH and osmolarity within the presently claimed ranges. The reference by Rota et al. clearly teaches that sodium lauryl sulfate (Equex STM paste) provides for higher motility and longer survival of frozen spermatozoa (abstract).

The semen extender composition as disclosed by Rota et al. contains an animal-derived egg yolk lecithin. However, EP 0 685 556 (Ghazarian) teaches substitution of a soy lecithin for the egg yolk preparation in the tris-glucose-citrate semen extender because egg yolk preparations might contain pathogenic microorganisms and because egg yolk preparations are fragile to sterilization and might loose their membrane protective effects upon sterilization or during storage (translation page 3). The semen extender of EP 0 685 556 (Ghazarian) is free from animal products and the amounts of ingredients are within the ranges of the claimed semen extender composition, for example: about 0.6-0.8% of phospholipids such as soy lecithin, about 0.5% of total carbohydrate, about 6-7% of freeze-agent or glycerol. Lecithin is also antioxidant. Although the cited patent is silent with regard to pH and osmolarity of the semen extender composition and/or solution for semen preservation, the values of pH and osmolarity that are

claimed are regular parameters that are commonly used for animal cell culture maintenance and preservation. The cited EP patent also teaches the method of making the semen extender composition by mixing the components of the composition. The cited EP patent also teaches incorporation of semen into the semen extender composition (example 3).

Although the cited EP 0 685 556 is lacking particular disclosure about the use of a sulfate-containing anionic surfactant. The reference by Rota et al. discloses a semen extender composition with 0.5% of an anionic sulfate-containing such as sodium lauryl sulfate (Equex STM paste. In addition, the reference by Hellmann et al. is relied upon for the teaching about the use of sodium lauryl sulfate in the composition intended for animal semen preservation (see English abstract) in amounts 0.2% (see OEP product in notes to figures 1 and 2). The reference teaches that incorporation of sodium lauryl sulfate had a significant effect on acrosome integrity of frozen and thawed semen (English abstract).

The composition of the cited EP 0 685 556 contains lecithin that is also antioxidant. Furthermore, it is well known to incorporate vitamins as antioxidants into semen extender compositions. For example: the cited patent US 6,130,034 (Aitken) teaches incorporation of antioxidant such as vitamin E, for example: see col. 1, line 50, as a commonly used and/or regular component in the composition intended for semen transportation and storage (col. 1, line 29). The suggested concentration for anti-oxidant vitamin E is 1mM (col. 1, line 54). Further, US 6,140,121 (Ellington et al]) also teaches incorporation of vitamins (entire document including col.17, line 11).

In addition, US 3,444,039 is relied upon to demonstrate that sodium citrate buffering preparation that is commonly used composition intended for semen preservation including semen

extender compositions of EP 0 685 556 and of the reference by Rota et al provides for neutral pH of about 6 -7 and osmolarity of about 250-300 mOsm which are regular pH and osmolarity parameters for animal cell culture maintenance and preservation (see col. 2, line 6 or see col. 3, line 30 and 44). And the cited US 6,140,121 (Ellington et al) teaches incorporation of various buffers into compositions intended for semen preservation including buffers such as EDTA (col. 19, line 28) or Tris or sodium citrate as well as surfactant (Tween 80) within the medium M199 in the composition intended for freezing sperm (col. 16, lines 57-59).

Therefore, it would have been obvious to one having ordinary skill in the art at the time the claimed invention was made to substitute a soy-derived lecithin of EP 0 685 556 for the egg yolk preparation in the tris-glucose-citrate semen extender with glycerol of Rota et al. because egg yolk preparations might contain pathogenic microorganisms and because egg yolk preparations are fragile to sterilization and might loose their membrane protective effects upon sterilization or during storage as taught by EP 0 685 556. It would also have been obvious to one having ordinary skill in the art at the time the claimed invention was made to add a sulfate-containing anionic surfactant or sodium lauryl sulfate and various antioxidants including vitamins to the soy-lecithin-tris-glucose-citrate semen extender of EP 0 685 556 with a reasonable expectation of success in obtaining composition suitable for semen maintenance and/or preservation because all claimed ingredients have been known and commonly used in the field of semen maintenance and preservation as adequately demonstrated by the cited references in combination. One of skill in the art would have been motivated to incorporate anionic surfactant such as sodium lauryl sulfate (Equex STM paste) into the semen extender preparations

for the expected benefits such as higher motility and longer survival of frozen spermatozoa as taught by Rota et al. and by Hellmann et al.

Thus, the claimed invention as a whole was clearly *prima facie* obvious, especially in the absence of evidence to the contrary.

The claimed subject matter fails to patentably distinguish over the state art as represented be the cited references.

Therefore, the claims are properly rejected under 35 USC § 103.

Response to Arguments

Applicants' arguments filed 10/23/2007 have been fully considered but they are not all found persuasive for the reasons below.

With regard to the claim rejected under 35 U.S.C. 112, *first paragraph*, (new matter) applicants appears to argue that the as-filed specification provides written support for the presently claimed sulfate containing anionic surfactant in the amounts about 0.0001% to about 1%. Yet, the full range as presently claimed relates to either to the use of a nonionic surfactant or at the very best to a mixture of several types of generic surfactants (page 6) without indicating a full range for a generic sulfate-containing anionic surfactant.

With regard to claim rejection under 35 USC § 103 applicants argue that there is no suggestion to combine references (response pages 9-13). However, the cited references are in the same field of endeavor (such as compositions intended for semen storage or preservation) and they seek to solve the same problems as the instant application and claims (such as provide for a

semen extender composition), and one of skill in the art is free to select components available in the prior art, *In re* Winslow, 151 USPQ 48 (CCPA, 1966).

In particular, with regard to EP 0 685 556 (Ghazarian) applicants argue that is fails to disclose the use of an "anionic surfactant". This argument is not found persuasive because the prior art as a whole recognizes incorporation of surfactants including incorporation specific anionic surfactant such as sodium lauryl sulfate into the semen extender composition as adequately taught by the reference by Hellemann et al., for example.

With regard to the cited patents US 3,444,039 (Rajamannan), US 6,130,034 (Aitken) and the reference by Hellmann et al applicants appear to argue that the cited compositions contain animal derived phospholipids from egg yolk. However, these prior art references are relied upon for the teaching about other than phospholipids components as explained above. Moreover, the cited US 6,140,121 (Ellington et al) and EP 0 685 556 (Ghazarian) clearly teach the exclusion of egg products since the animal products including egg products could carry pathogens. For example: see US 6,140,121 at col. 27, line 16-30. The cited EP 0 685 556 discloses compositions with non-animal derived phospholipids such as soy lecithin and the cited US 6,140,121 (Ellington) clearly suggests incorporation of soy lecithin as alternative to egg yolk for the non-egg yolk containing semen extenders (col. 27, lines 20-30).

Motivation to combine the prior art teaching can come not only from direct teaching of the prior art, but also the nature of the problem to be solved and/or the knowledge of persons of ordinary skill in the art, Ruiz v. A.B. Chance Co. 357 F.3d 1270, 69 USPQ2d 1686 (2004). Further, the examiner recognizes that references cannot be arbitrarily combined that there must be some reason why one skilled in the art would be motivated to make the proposed combination

of primary and secondary references, *In re* Nomiya, 184 USPQ 607 (CCPA 1975). However, there is no requirement that a motivation to make the modification be expressly articulated. One test for combining references is what the combination of disclosures taken as a whole would suggest to one versed in the art, rather than by their specific disclosures, *In re* Bozek, 163 USPQ 545 (CCPA 1969). In this case, the use of components known in the art, and used for their known art specific properties even in different combinations, is considered to be obvious in the absence of evidence to the contrary.

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Vera Afremova whose telephone number is (571) 272-0914. The examiner can normally be reached from Monday to Friday from 9.30 am to 6.00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Jon P. Weber, can be reached at (571) 272-0925.

The fax phone number for the TC 1600 where this application or proceeding is assigned is (571) 273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Technology center 1600, telephone number is (571) 272-1600.

Vera Afremova

AU 1657

January 14, 2008

VERA AFREMOVA

PRIMARY EXAMINER

Notice of References Cited	Application/Control No. 10/044,796	Applicant(s)/Patent Under Reexamination LOSKUTOFF ET AL.	
	Examiner Vera Afremova	Art Unit 1657	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
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	L	US-			
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Rota et al. "Fertility after vaginal or uterine deposition of semen frozen in a tris extender with or without STM Paste". Theriogenology, (April 15, 1999) Vol.51, No. 6, pages 1045-1058.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

AB Twenty-five bitches were artificially inseminated with semen that was frozen-thawed using an egg yolk-Tris-glucose-citrate extender containing 5% glycerol with, or without the addition of 0.5% Equex STM Paste. Semen was collected on 2 occasions from 11 dogs, pooled, and evaluated for sperm motility, morphology and plasma membrane integrity. Each pool was then divided in 2 parts, diluted with 1 of the 2 extenders, and frozen in 0.5-mL straws. In the bitches, plasma progesterone was assayed daily during late proestrus and estrus. Artificial insemination (AI) was performed twice on Days 3 and 5 after the estimated LH peak. For each insemination, 200X106 spermatozoa were used. Ten bitches were inseminated with semen frozen without Equex: In 5 females, semen was deposited transcervically into the uterus with the aid of a fiberoptic endoscope and a urethral catheter, while the remaining 5 bitches were inseminated in the cranial vagina using a Norwegian catheter. Fifteen bitches were inseminated with semen frozen-thawed with Equex: Two groups of 5 bitches were inseminated according to the techniques described above, while 5 bitches were inseminated vaginally using the Osiris catheter. Pregnancy was diagnosed and the number of fetuses counted by ultrasound examination. Post-thaw, spermatozoa frozen with Equex tended to have higher total and progressive motility and to survive longer in vitro than when the extender without Equex was used. Spermatozoal concentration, age of the bitches, duration of heat and estrus, and progesterone concentration at LH peak and at the first and second AI did not differ among the 5 groups. The overall pregnancy rate of 84% (21/25) was close to what can be expected from well controlled natural matings. For both freezing extenders tested, 5/5 bitches were pregnant after uterine deposition of semen and 4/5 were pregnant when semen was deposited in the anterior vagina using the Norwegian catheter. With the Osiris catheter, 3/5 inseminations resulted in a pregnancy. No significant differences in pregnancy rate of number of fetuses were found between groups, site of deposition of freezing extender.

ACCESSION NUMBER: 1999:307796 BIOSIS

TITLE: Fertility after vaginal or uterine deposition of dog semen frozen in a tris extender with or without Equex STM Paste.

AUTHOR(S): Rota, A. [Reprint author]; Iguer-Ouada, M.; Verstegen, J.; Linde-Forsberg, C. [Reprint author]

SOURCE: Theriogenology, (April 15, 1999) Vol. 51, No. 6, pp. 1045-1058.

ELSEVIER

FERTILITY AFTER VAGINAL OR UTERINE DEPOSITION OF DOG SEMEN FROZEN IN A TRIS EXTENDER WITH OR WITHOUT EQUEX STM PASTE

A. Rota,[1] M. Iguer-Ouada,[2] J. Verstegen[2] and C. Linde-Forsberg[1]

[1]Department of Obstetrics and Gynaecology, Centre for Reproductive Biology
Swedish University of Agricultural Sciences, Faculty of Veterinary Medicine, Uppsala, Sweden;
[2]Department of Small Animal Reproduction, University of Liège, Liège, Belgium.

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ABSTRACT

Twenty-five bitches were artificially inseminated with semen that was frozen-thawed using an egg yolk-Tris-glucose-citrate extender containing 5% glycerol with, or without the addition of 0.5% Equex STM Paste. Semen was collected on 2 occasions from 11 dogs, pooled, and evaluated for sperm motility, morphology and plasma membrane integrity. Each pool was then divided in 2 parts, diluted with 1 of the 2 extenders, and frozen in 0.5-mL straws. In the bitches, plasma progesterone was assayed daily during late proestrus and estrus. Artificial insemination (AI) was performed twice on Days 3 and 5 after the estimated LH peak. For each insemination, $200x10^6$ spermatozoa were used. Ten bitches were inseminated with semen frozen without Equex: In 5 females, semen was deposited transcervically into the uterus with the aid of a fiberoptic endoscope and a urethral catheter, while the remaining 5 bitches were inseminated in the cranial vagina using a Norwegian catheter. Fifteen bitches were inseminated with semen frozen-thawed with Equex: Two groups of 5 bitches were inseminated according to the techniques described above, while 5 bitches were inseminated vaginally using the Osiris catheter. Pregnancy was diagnosed and the number of fetuses counted by ultrasound examination. Post-thaw, spermatozoa frozen with Equex tended to have higher total and progressive motility and to survive longer in vitro than when the extender without Equex was used. Spermatozoal concentration, age of the bitches, duration of heat and estrus, and progesterone concentration at LH peak and at the first and second AI did not differ among the 5 groups. The overall pregnancy rate of 84% (21/25) was close to what can be expected from well controlled natural matings. For both freezing extenders tested, 5/5 bitches were pregnant after uterine deposition of semen and 4/5 were pregnant when semen was deposited in the anterior vagina using the Norwegian catheter. With the Osiris catheter, 3/5 inseminations resulted in a pregnancy. No significant differences in pregnancy rate or number of fetuses were found between groups, site of deposition or freezing extender.

Key words: dog, frozen semen, artificial insemination, fertility

Acknowledgements

The authors thank the Department of Small Animal Clinical Sciences, SLU, for allowing semen collection from their dogs.

INTRODUCTION

Whelping rate after well-controlled natural matings in the dog varies between 85 and 90% (7, 11). The whelping rate after artificial insemination (AI) with frozen-thawed semen is usually lower, and litter size is smaller (18, 20, 21, 40), although in a number of controlled studies employing a single freezing-thawing technique, it was close to that obtained after natural mating (4, 15, 26, 47).

Several factors affect the outcome of AIs when using frozen-thawed semen. Post-thaw semen quality and spermatozoal numbers are important, and, in turn, depend on the quality of ejaculated semen, its freezability and techniques used for the freezing-thawing process. The recommended number of dog spermatozoa to be used for each insemination with frozen-thawed semen is 150 to $200x10^6$ cells. The number of bitches that can be inseminated with 1 ejaculate, therefore, ranges between 1 and 5. Timing of the inseminations is of great importance, particularly when frozen-thawed semen is used. Because of spermatozoal longevity, natural matings may be successful when performed several days before the fertilization period. However, frozen-thawed semen must be inseminated when the oocytes are ready to be fertilized. In the dog, primary oocytes are ovulated 24 to 78 h after the LH surge, and require approximately 2 to 3 more days to mature to Metaphase II (22, 30, 48). Therefore, AI using frozen-thawed semen should be performed 3 to 7 d after the LH peak. Peripheral progesterone concentrations can be used to time the events related to the LH surge and ovulation in this species (19).

Vaginal insemination is easy to perform but when used for frozen-thawed semen it yields lower pregnancy rates than intrauterine insemination (13, 15). Deposition of semen in the anterior vagina can be performed using a disposable bovine uterine catheter (19), the Norwegian catheter (12), or the Osiris catheter with a balloon on its tip, which can be inflated to prevent the reflux of semen (23). Uterine insemination can be performed transcervically using the Norwegian catheter (3) or with the aid of a fiberoptic endoscope and a urethral catheter (47). Laparotomy or laparoscopy might also be used, although this entails increased levels of stress for the bitch and higher costs for the owner (2, 39).

One reason for the generally lowered fertility of cryopreserved semen in this species is considered to be the short life span of frozen-thawed spermatozoa, thus the need for perfect timing and uterine deposition of the semen (19, 24). Incubation of thawed spermatozoa at body temperature partially mimics the situation in vivo, and repeated evaluations of motility under these conditions have therefore been used to test semen quality in the dog (8, 9, 27, 35, 43). In a previous study, we found that the addition of Equex STM Paste to a freezing extender improved the longevity in vitro of frozen-thawed dog spermatozoa (35). Equex STM Paste is a commercially available additive for use with semen extenders. It contains sodium dodecyl sulphate (SDS), a detergent thought to act through an alteration of the egg yolk, possibly increasing its protecting potential against cold shock and freezing injury (31). Whether the addition of Equex STM Paste to the freezing extender also increases pregnancy rates after AI with frozen-thawed semen has not been studied in the dog.

The aim of this study was to determine if the addition of Equex STM Paste to the freezing extender increases pregnancy rates in bitches after vaginal and uterine deposition of frozen-thawed spermatozoa.

MATERIALS AND METHODS

Animals

This study was conducted in accordance with the Swedish and Belgian legal requirements for animal welfare and experimentation, and the National Institute of Health guide for animal care and experimentation.

Fourteen male dogs, of different breeds and ages (average: 4 yr; range 1.5 to 8 yr), were included in this study. Motility of each semen sample was estimated to be above 75%. Ten of the dogs were of different breeds and were privately owned, and 4 were beagles that belonged to the Department of Surgery and Medicine at the Faculty of Veterinary Medicine, SLU, Uppsala, Sweden.

Twenty-five beagle bitches in which the previous cycles had been characterized were used for the study. All bitches had been pregnant at least once. The average age was 4.7 yr and ranged from 2 to 9 yr. The bitches were located at the Department of Small Animal Reproduction, University of Liège, Belgium, and were kept in groups of 2 to 3 in kennels 1.5x2-m. The bitches had access to outdoor runs for at least 2 h per day, and were fed Hill's canine maintenance science plan diet in the amount suggested by the manufacturer; water was available ad libitum.

Diluents and Extenders

Two freezing extenders were prepared as a single batch, as described by Rota et al. (35), and stored frozen. In brief, the extenders were Tris-glucose-citrate buffers with 20% egg yolk and 5% glycerol, with (EYT-GE) or without (EYT-G) 0.5% Equex STM Paste.

A Tris diluent (TRIS buffer), containing 2.4 g Tris, 1.4 g citric acid mono-hydrate, 0.8 g glucose, 0.06 g Na-benzylpenicillin and 0.1 g streptomycin sulphate solubilized in 100 mL of distilled water, was also prepared and stored frozen (35). It was used to dilute 1:3 the frozen semen after thawing, prior to evaluation and insemination.

Semen Evaluation

Morphology. From each pool, smears of the fresh, undiluted semen were made for sperm head morphology assessment (stained with carbol-fuchsine stain; 46). An aliquot was fixed in buffered formol-saline (5) to be evaluated for tail, midpiece and acrosome defects.

Motility. Motility of fresh semen, was assessed subjectively under a phase contrast microscope at 37°C and x 400 magnification. Two aliquots from each semen sample were placed on a glass slide under a coverslip, and at least 8 fields were examined in each subsample. Two

straws for each diluent and pool were evaluated for motility in the same manner, immediately after thawing and dilution 1:3 in the TRIS buffer, and again after 1, 2 and 3 h of incubation at 38°C. Prior to each insemination, a 10-µL aliquot of frozen/thawed semen was placed in a Makler chamber (Sefi-Medical Istruments, Haifa, Israel) at 38°C and evaluated for sperm concentration, motility and progressive motility using the Hamilton Thorn Motility Analyzer (IVOS version 10, Hamilton Thorn Research Danvers, MA, USA) under 1.95 x 10 phase contrast magnification. Five fields randomly selected by the computer were analyzed for each semen sample. The settings were the following: frames aquired 30, frame rate 60 Hz, minimum contrast 75, minimum static contrast 30, straightness threshold 75%, low VAP cut-off 9.9 µm/sec, medium VAP cut-off 50 µm/sec, head size nonmotile 6 pix, head intensity nonmotile 140, static head size 0.48 to 2.76, static head intensity 0.35 to 1.27.

Plasma membrane integrity. The fresh semen, and 2 straws for each diluent and pool immediately after thawing and dilution 1:3 in the TRIS buffer, and again after 1, 2 and 3 h of incubation at 38°C were also evaluated for sperm plasma membrane integrity. This was assessed using 6-carboxyfluorescein diacetate and propidium iodide (C-FDA/PI) fluorophores (16, modified; 34). For each sample stained with C-FDA/PI, 100 sperm cells were counted on 2 slides (total=200) and classified as having an intact plasmalemma (IPM) when stained green with C-FDA and unstained with PI, and as having a damaged plasmalemma when the cells stained red with PI.

Semen collection, dilution, freezing and thawing

On 2 occasions, 1 wk apart, 1 ejaculate was obtained from 11 dogs. Eight dogs were donors on both occasions, while 6 dogs were donors on one occasion only. The sperm-rich fraction of the ejaculate was collected in a calibrated plastic vial by digital manipulation (19). The volume was measured and motility evaluated. The 11 semen samples were pooled together to avoid an effect of dog on the outcome of insemination. Pooled sperm concentration was measured by a photometer (Spermacue®, Minitüb, Tiefenbach, Germany). Pool volume and pool sperm motility were assessed, and smears were made for assessment of morphology. Before evaluation of plasma membrane integrity, subsamples were diluted 1:3 in TRIS buffer.

Aliquots of semen each containing $1x10^9$ spermatozoa were centrifuged at 700g for 8 min. The supernatant was discarded, and the pellets were diluted in 2 steps with the freezing extenders, as described by Rota et al. (35) to a final estimated sperm concentration of $200x10^6$ cells/mL. After 70 to 140 min from the onset of cooling, the extended semen was frozen in 0.5-mL straws by lowering them, in 3 steps, into 2 Apollo SX-18 LN_2 tanks (MVE Cryogenetics®, New Prague, MN, USA; 15, modified). Before introducing the goblets and beginning the freezing process, the empty canister was kept in the neck of the LN_2 tank for 5 min (43). Each pool was frozen over several operations, as a maximum of 16 straws (4 straws in each goblet) per operation were frozen, in order to standardize the freezing rate. Each straw was thawed in a waterbath at 38°C for 1 min and emptied into a tube. Thawed semen was then diluted 1:3 with TRIS buffer, at 38°C.

Estrus detection

All bitches were examined once a day for the presence of vulval swelling and serosanguineous discharge, indicating the onset of proestrus. The estrus cycle was monitored daily by vaginal smears from onset of proestrus until the first day of cytological diestrus (37). Plasma progesterone was measured daily from mid proestrus to diestrus, and then 3 times a week until pregnancy was diagnosed.

The day of the LH peak was estimated to be the day when progesterone concentrations reached values between 2.5 and 10 nmol l^{-1} (6). The duration of heat was defined as the period (in days) from the first day of vulval swelling and detection of a serosanguineous vaginal discharge to the first day of cytological diestrus. The duration of estrus was defined as the interval (in days) from the day of the estimated LH peak to the first day of cytological diestrus.

Blood samples and hormonal analyses

Blood was collected by jugular venipuncture into heparinized tubes. Samples were centrifuged and plasma stored at -20°C until assayed for progesterone. Progesterone concentrations were measured by Radioimmunoassay (Progesterone Coat-a-Count kit, Diagnostic Products Corporation, Los Angeles, CA, USA) validated for dog plasma (42).

Artificial Inseminations

The 25 bitches were assigned to 5 treatment groups, each consisting of 5 bitches: Group 1, uterine deposition, transcervical, semen frozen with EYT-GE; Group 2, vaginal deposition, Norwegian catheter, semen frozen with EYT-GE; Group 3, vaginal deposition, Osiris catheter, semen frozen with EYT-GE; Group 4, uterine deposition, transcervical, semen frozen with EYT-G; Group 5, vaginal deposition, Norwegian catheter, semen frozen with EYT-G.

Two inseminations were performed on each bitch at 3 and 5 d after the day of the estimated LH peak. Two straws were used for each AI. The bitches were inseminated with 2 straws from Pool 1, and 2 straws from Pool 2, to minimize any possible effect of differences between the freezing batches.

Intrauterine deposition was performed transcervically using the technique developed by Wilson (47). A rigid urethero-renoscope (Karl Storz Model 27024 KB, Karlsruhe, Germany, external diameter 0.3 cm) connected to an electronic light source (Electronic Light Source 5000 M Endotron 220 V, Amsterdam, Holland) and to a video camera (Scoopman 504 Moritex, Tokyo, Japan) was introduced in the CO_2-inflated vagina until the cervix was visualized. A semi-rigid sterile male dog urinary catheter (N°4, Rusch AG, 71394 Kernen, Germany) was introduced through the endoscope and the cervix, into the body of the uterus. After insemination, the endoscope and catheter were removed, and the bitch was kept for 10 min with elevated hindquarters.

Two techniques were used for vaginal inseminations. The first one utilized the inner metallic part of a Norwegian catheter (Norske Pelsdyrforlag A/L, Oslo, Norway). The bitch was

held with the hindquarters elevated, the catheter was introduced to the cranial vagina, and the semen was deposited. After deposition, the catheter was withdrawn and the bitch held with elevated hindquarters for 10 min to prevent reflux of semen. The second technique for vaginal insemination used the French Osiris catheter (23 - IMV L'Aigle France). After introduction of the catheter into the cranial vagina, the balloon was inflated with 7 to 20 mL air and the semen deposited. After insemination, the bitch was kept for 10 min with elevated hindquarters before removal of the catheter.

Pregnancy diagnosis and induction of abortion

Pregnancy was detected by ultrasonography (Hitachi EUB 415, B&M Mode, 7.5 Mhz linear probe, Tokyo, Japan) 4 times a week from Day 15 post the LH surge until Day 30. The number of fetuses was counted and recorded. At Day 28 of pregnancy abortion was initiated, using the protocol described by Onclin and Verstegen (29).

Statistical analyses

Results are presented as means and standard deviations. Analysis of variance was used to evaluate differences between the 5 groups, when the residuals were found to be normally distributed by the Ryan Joiner goodness-of-fit test. For sperm concentration, motility and progressive motility, differences between extenders were evaluated by the Wilcoxon Signed Rank Sum Test, as were the differences in number of fetuses between site of semen deposition and freezing extender used. Differences in number of fetuses among the 5 groups was evaluated using the Kruskall-Wallis test. The analyses were performed using the Minitab Statistical software (State College, PA, USA). Differences in pregnancy rates among the 5 groups, site of semen deposition and extender, were evaluated using the Fisher's exact test in the SAS software package (Cary, NC, USA) (1).

RESULTS

Semen Quality

The total number of spermatozoa collected to create Pools 1 and 2 were 6.721×10^9 and 10.120×10^9 cells, respectively. Motility and the proportion of spermatozoa showing an intact plasma membrane (IPM) were ≥80% and morphologically normal spermatozoa were ≥75% in both pools of fresh semen (Table 1).

After freezing and thawing, mean motility immediately post-thawing, and after 1, 2 and 3 h of incubation at 38°C was 47, 21, 17 and 14% for semen frozen with EYT-G, and 54, 44, 42 and 39% for semen frozen with EYT-GE. Mean proportion of spermatozoa with an intact plasma membrane immediately post-thawing, and after 1, 2 and 3 h of incubation at 38°C was 64, 29, 22 and 23% for semen frozen with EYT-G, and 60, 50, 50 and 49% for semen frozen with EYT-GE.

Table 1. Motility and the proportion of morphologically normal and plasma membrane-intact spermatozoa in fresh semen samples from the pooled semen.

	Sperm motility %	Morphologically normal spermatozoa %	Plasma membrane-intact spermatozoa %
Pool 1	80	76	84.5
Pool 2	85	78	89.5

Concentration of spermatozoa and post-thaw total and progressive motility evaluated by the Hamilton Thorne Motility Analyzer did not differ among the 5 groups (ANOVA; P>0.1). When the straws that were used for AI were compared between extenders, spermatozoal concentration did not differ (Wilcoxon Signed Rank Sum Test, corrected for ties; P>0.4), but there was a tendency (Table 2) for post-thaw motility and progressive motility to be higher in the EYT-GE extender (Wilcoxon Signed Rank Sum Test, corrected for ties; P=0.053 and P=0.062, respectively).

Table 2. Spermatozoal concentration and total and progressive sperm motility of the frozen-thawed semen diluted in either of 2 extenders and used for artificial insemination in the 5 groups of bitches (Mean ± SD).

Treatment groups	Sperm concentration (10^6/mL)	Sperm motility (%)	Sperm progressive motility (%)
EYT-G (intrauterine)	241 ± 24	61 ± 13	34 ± 11
EYT-G (intravaginal)	262 ± 29	58 ± 9	30 ± 16
EYT-GE (intrauterine)	249 ± 20	63 ± 5	33 ± 10
EYT-GE (intravaginal)	259 ± 28	69 ± 10	37 ± 16
EYT-GE (Osiris[a])	250 ± 8	69 ± 4	39 ± 19
EYT-G[b] extender	252 ± 27	59 ± 11	32 ± 6
EYT-GE[c] extender	253 ± 19	67 ± 7	36 ± 5

[a] Osiris = intravaginal with Osiris catheter.

[b] EYT-G = Egg yolk Tris glycerol.

[c] EYT-GE = Egg yolk Tris glycerol Equex.

Bitches

Neither age, duration of heat, or duration of estrus differed among the 5 treatment groups (ANOVA; P>0.2), nor did serum progesterone concentrations on the day of the estimated LH peak or on the days of the first and second AI (ANOVA; P>0.3; Table 3).

Pregnancy rates and number of fetuses

The overall pregnancy rate was 84% (21/25). For both freezing extenders tested, 5/5 bitches were pregnant after uterine deposition of semen, and 4/5 after deposition with the Norwegian catheter into the anterior vagina. When semen was frozen with the EYT-GE extender and deposited in the vagina with the Osiris catheter, 3/5 bitches conceived (Table 4). No significant differences were found among the 5 groups (Fisher's exact test P=0.753), between the sites of deposition (uterine vs vaginal, showing pregnancy in 10/10 vs 11/15 bitches, respectively, Fisher's exact test P=0.125) or the freezing extenders (EYT-G vs EYT-GE showing pregnancy in 9/10 vs 12/15 bitches, respectively, Fisher's exact test, P= 0.626).

Table 3. Age, heat, estrus and plasma progesterone (P4) concentration (nmol l^{-1}) at the time of estimated LH peak, first AI and second AI for the 5 treatment groups. Mean ± SD (range).

Treatment groups	EYT-G [a] (intrauterine)	EYT-G [a] (intravaginal)	EYT-GE [b] (intrauterine)	EYT-GE [b] (intravaginal)	EYT-GE [b] (Osiris [c])
Age (years)	3.9 ± 2.1 (1.5-6)	4.3 ± 3.4 (1.5-8)	5.9 ± 3.8 (1.5-9)	3.3 ± 1.8 (1.5-6)	4.8 ± 2.3 (2-8)
Length of heat (days)	15.8 ± 0.8 (15-17)	15.4 ± 0.8 (13-17)	15.8 ± 0.5 (14-20)	15.4 ± 0.1 (14-17)	15.4 ± 0.1 (13-16)
Length of estrus (days)	9.4 ± 1.7 (8-12)	8.4 ± 1.5 (6-10)	9.0 ± 0.7 (8-10)	8.4 ± 1.1 (7-12)	7.6 ± 0.5 (7-8)
P4 on estimated day of LH peak	7.1 ± 2.7 (2.9-9.9)	5.5 ± 0.3 (5.1-5.7)	5.5 ± 1.4 (3.2-7.0)	5.5 ± 1.8 (2.9-7.6)	5.5 ± 1.8 (3.5-7.9)
P4 on day of first AI	21.1 ± 4.9 (17.5-29.6)	22.6 ± 5.2 (16.8-28.9)	25.9 ± 5.9 (17.5-31.5)	20.3 ± 4.8 (12.7-24.5)	25.1 ± 12.1 (10.1-43.9)
P4 on day of second AI	54.8 ± 11.5 (40.1-69.0)	55.6 ± 16.1 (32.1-74.1)	45.6 ± 10.1 (32.4-56.9)	41.5 ± 16.5 (31.8-70.9)	59.7 ± 18.7 (30.2-75.4)

[a] EYT-G = Egg yolk Tris glycerol.
[b] EYT-GE = Egg yolk Tris glycerol Equex.
[c] Osiris = intravaginal with Osiris catheter.

Table 4. Number of pregnant bitches in the 5 treatment groups

Pregnancy status	EYT-G[a] intrauterine	EYT-G[a] intravaginal	EYT-GE[b] intrauterine	EYT-GE[b] intravaginal	EYT-GE[b] Osiris[c]	Total no. of bitches
Pregnant	5	4	5	4	3	21
Not pregnant	0	1	0	1	2	4
Total no. of bitches	5	5	5	5	5	25

[a] EYT-G = Egg yolk Tris glycerol.
[b] EYT-GE = Egg yolk Tris glycerol Equex.
[c] Osiris = intravaginal with Osiris catheter.

The number of fetuses counted on Day 30 of pregnancy did not differ significantly among the 5 treatment groups (Kruskall-Wallis test, corrected for ties; P>0.2), between the 2 sites of deposition (uterus or vagina), or between the 2 types of extenders used for freezing of the semen (Wilcoxon Signed Rank Sum Test, corrected for ties; P>0.1 and P>0.4, respectively; Table 5).

Table 5. Number of fetuses counted by ultrasound on Day 30 of pregnancy in the 5 treatment groups of bitches.

Treatment groups	EYT-G [a] (intrauterine)	EYT-G [a] (intravaginal)	EYT-GE [b] (intrauterine)	EYT-GE [b] (intravaginal)	EYT-GE [b] (Osiris [c])
Fetuses					
mean ± sd	3.6 ± 0.5	4.7 ± 0.5	3.2 ± 2.0	3.5 ± 1.3	2.0 ± 1.7
n	5	4	5	4	3

[a] EYT-G = Egg yolk Tris glycerol.
[b] EYT-GE = Egg yolk Tris glycerol Equex.
[c] Osiris = intravaginal with Osiris catheter.

DISCUSSION

An overall pregnancy rate similar to that after well-controlled natural matings was obtained in 25 bitches inseminated with frozen-thawed semen. When the semen was deposited in the uterus, 10/10 (100%) bitches became pregnant. The pregnancy rate after vaginal deposition of semen, though lower (11/15; 73%) did not differ significantly. There was no significant difference in the pregnancy rate between the 2 extenders.

Although the first reported (38) pregnancies using frozen-thawed semen for AI in the dog were obtained with vaginal deposition of the semen, Andersen (2) failed to obtain pregnancies when depositing frozen-thawed semen at this site, but was successful when placing the insemination dose into the uterus. Vaginal deposition of frozen-thawed semen has been found to lower whelping rates compared with intrauterine deposition (13, 15, 20), possibly due to a decrease in spermatozoal longevity after thawing. The 2 extenders tested (EYT-G and EYT-GE) differed significantly in terms of post-thaw viability and longevity in vitro, as defined by the proportion of motile and plasma-membrane-intact spermatozoa during 3 h of incubation at 38°C (35). In other species, estimates of motility after incubation at body temperature were found to give a better indication of fertilizing ability than evaluations immediately post thawing (17, 36). It has been speculated that sperm survival in vitro would be an important parameter for measuring the fertilizing ability of canine spermatozoa. In the present study, however, no such relationship was found, possibly due to the number of spermatozoa used in this trial.

When estimating the number of spermatozoa needed for optimal fertilization in the dog, Tsutsui et al. (44) found a significant reduction in the rate of pregnancy after a single vaginal deposition using $100x10^6$ rather than $200x10^6$ viable, fresh spermatozoa, but the litter size did not differ. No pregnancies were obtained using the $25x10^6$ sperm dose; however, the volume of the inseminate (1 or 3 mL) had no effect on the results. When fresh semen was deposited

surgically at the tip of 1 uterine horn, $10x10^6$ spermatozoa resulted in a high pregnancy rate (91%), although $20x10^6$ spermatozoa were needed for the fertilization of oocytes from both ovaries (45). Since frozen-thawed spermatozoa are thought to have reduced viability and fertilizing capacity, a higher number of cells in the AI-dose would probably be needed to obtain the same pregnancy rates as with fresh semen. The number of spermatozoa used in the present study ($200x10^6$ per AI) is in the range recommended for intrauterine deposition of frozen-thawed semen under clinical conditions (4, 24), and this dosage might be necessary to obtain the maximum conception rate and litter size with commonly used semen cryopreservation methods. If the proportion of spermatozoa that remain viable and having fertilizing capacity post thawing were to be increased by improved freezing methods, then the total number of spermatozoa needed to obtain the same pregnancy rates would be lower. The benefits obtained would be masked in a fertility trial if the number of spermatozoa used for AI is not diminished.

To obtain maximum conception rates in the dog, the insemination dose for vaginal deposition of fresh semen has been shown to be 10-fold higher than for intrauterine insemination (44, 45). Lees and Castleberry (18) and Olar et al. (28) used 100 to $150x10^6$ motile frozen-thawed spermatozoa per inseminate and multiple (3 to 9) vaginal inseminations in each cycle, resulting in 8/14 (57%) and 3/12 (25%) bitches being pregnant, respectively. Gill (14) achieved no conceptions in a total of 12 bitches after 3 vaginal AIs/cycle of about $60x10^6$ motile frozen-thawed spermatozoa each. However, the frozen-thawed semen of a single dog was shown to be repeatedly fertile when $30\text{-}35x10^6$ viable, normal spermatozoa were deposited into the uterus on 2 occasions during estrus (47). Nöthling (25) obtained pregnancy rates of approximately 90% after multiple (average: 5.3, range 1 to 12) vaginal inseminations with varying numbers of frozen-thawed sperm cells (9 to $300x10^6$cells/AI), and concluded that timing of insemination seemed to be more important than the sperm number per AI for the range of sperm numbers used in their study.

Timing of AI was done through measurement of peripheral plasma progesterone concentrations and subsequent estimation of the day of the LH peak. Raise of plasma progesterone concentrations during estrus and diestrus varies among bitches, but the day of the first significant increase can be considered as the day of the LH peak (6). The measurement of progesterone by radioimmunoassay to estimate the day of LH peak has been reported previously (32, 41). In a clinical study it was found that the chances of fertilization with frozen-thawed spermatozoa in the dog were higher if AI was performed when the plasma progesterone concentration was above rather than below 30 nmol l^{-1} (20). Most of the early workers did not have this tool for the timing of AI, and this might have affected their results, although doing multiple inseminations during the estrus period would have compensated for this. In the present study the timing to AI was accurate, and this could have masked the possibility that a higher proportion of spermatozoa may remain viable post thawing when the extender contains Equex STM paste.

Heterospermic insemination (i.e. pooling the semen from 2 or more males) was used in order to have a large homogeneous sample to inseminate all 25 bitches. In the bull, the use of single breed heterospermic insemination from three males did not significantly increase conception rate (33). It has not been established if mixing ejaculates from several dogs might improve conception rates. However, when both pooled and non pooled frozen-thawed semen

samples were used in the same study, no difference in pregnancy rates was observed (40). On the contrary, the inclusion in the pool of one or more ejaculates of low quality, could decrease the overall fertilizing potential of the same pool.

Silva et al. (40) obtained equal pregnancy rates using the Osiris catheter (i.e. vaginal AI) and laparoscopic intrauterine deposition of fresh (5/5) and frozen-thawed semen (6/10). In that study vaginal deposition was achieved using either the Norwegian or the Osiris catheter and no advantage was seen by using the latter. The Osiris catheter is single-use and costly, while the Norwegian catheter can be sterilized and re-used. The Norwegian catheter is not considered to be suitable for vaginal AI because of the possibility of reflux of semen from the vagina, between the plastic sheet and the steel catheter (12). This effect was avoided in this study, by using only the inner steel part of the catheter, which permits deposition of semen in proximity of the cervix.

Pregnancies and number of fetuses were evaluated by ultrasound examination. Although this method allows for early pregnancy detection (17 to 20 d after the LH peak, gestational sacs may be detected, and at 23 to 25 d the embryonic mass and heartbeats can be seen), the accuracy of predicting fetal number is low; it was found to be 38% during early pregnancy (20 to 30 d after cytological metestrus) and to be even lower with later examinations (10, 49, 50). For this reason, some uncertainty exists as to actual number of conceptuses in this study.

In conclusion, with the combination of techniques available for both freezing-thawing of semen and monitoring of the cycle, it is possible to obtain pregnancy rates close to what can be expected from well-controlled natural matings. The minimum sperm number needed to obtain maximum fertility after frozen-thawed semen AI in this species remains to be determined.

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RALPH
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May 27, 2004

Re: RMTC Job No. 1604-98343

To Whom It May Concern:

This is to certify that a professional translator on our staff who is skilled in the **French** language translated the document(s) noted below from **French** into **English**.

- European Patent 0 685 556 A1

We certify that the attached **English** translation conforms essentially to the original **French** language.

Kim Vitray
Operations Manager

Subscribed and sworn to before me this 27TH day of MAY 2004.

TINA WUELFING
Notary Public
State of Texas
My Commission Expires
December 8, 2007

Tina Wuelfing
Notary Public

910 WEST AVE.
AUSTIN, TEXAS 78701
www.mcelroytranslation.com

(512) 472-6753
1-800-531-9977
FAX (512) 472-4591

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AQUEOUS VEHICLE FOR NONAUTONOMOUS MICROORGANISMS OF THE ANIMAL KINGDOM TO BE KEPT ALIVE OUTSIDE THEIR NATURAL ENVIRONMENT, ESPECIALLY BOVINE SPERMATOZOA

Inventors:

Chaqué Ghazarian
5 route de Beaulieu
F-69210 Saint-Bel, France

Bertrand Cassou
Saint Symphorien des Bruyeres
F-61300 L'Aigle, France

Serge Bousseau
Le Parc
F-61300 L'Aigle, France

Jean-Pierre Brillard
45, rue du Val Violet
F-37300 Joue Les Tours, France

Applicant:

Instruments de Médicine Vétérinaire
10 rue Georges Clemenceau
F-61300 L'Aigle, France

Agent: Bonnet-Thirion Law Firm
95 Boulevard Beaumarchais
F-75003 Paris, France

The vehicle of the invention is designed for nonautonomous microorganisms of the animal kingdom such as gametes or embryos that need to be kept alive outside their natural environment with a view to human interventions, and is capable of being stored ready to use over prolonged periods. It concerns an aqueous medium that comprises, especially beside nutrition agents (sugars and amino acids), buffers and mineral salts usually used, a protective product formed as support for embryo growth by a living organism, which, in the state of the technique is of animal origin and is added immediately before use of the vehicle and which, according to the invention, is a lecithin extracted from soy seeds and introduced into the aqueous medium upon formulation of the vehicle. When the storage of the microorganisms is cryogenic, glycerol is added to the vehicle. In a specific application, the microorganisms are bovine spermatozoa.

The invention is related to a vehicle for nonautonomous microorganisms of the animal kingdom, such as gametes and embryos, for keeping them alive outside their natural environment with a view to human intervention, capable of being stored ready to use over prolonged periods, this vehicle being an aqueous medium comprising, especially beside nutrition agents, buffers and mineral salts, a protective product formed as support for embryonic growth by a living organism and which, in the state of the technique is of animal origin and added immediately before use of the vehicle.

In the sense of the present description, nonautonomous microorganism is understood to be unicellular or paucicellular, eukaryotes or prokaryotes, living but without the ability for immediate reproduction; that is, for reproduction of descendent organisms at the same stage of evolution as their direct ancestors. Thus, gametes or embryos are not autonomous because they cannot directly create gametes or embryos. On the other hand, amebas form autonomous microorganisms.

The storage of living gametes, embryos and cells of the animal kingdom outside their natural environment in the fresh state and especially frozen for veterinary or closely related interventions, especially for artificial fertilization, requires liquid vehicles which in terms of the expert are called "diluents" or "preservatives" for spermatozoa and media for oocytes, embryos and cells. These liquids include in aqueous media, nutrition agents such as sugars and amino acids, buffers and possibly anticoagulants, as well as, for cryogenic storage of microorganisms, an inhibitor of ice crystal formation such as a polyol, especially glycerol. To it is added at the time of use a protective product for cell membranes that is of animal origin such as serums and

albumins; very often this protective product for the diluents is egg yolk, the protective ability of which is particularly accentuated because, it seems, that this egg yolk forms the essential support of growth of the chicken embryo; therefore, of a fragile organism at a stage where cell division is particularly intense, and the defense reactions to external injury even less developed. In practice, either fresh egg yolk or industrial liquid preparations designed especially for the food industry are used.

In compensation for the effectiveness of membrane protection, these protective products are favorable for the multiplication of microorganisms that may or may not be pathogenic, their storage in the effective state is temporary such that they are systematically added to the vehicle only at the time of use.

But this addition at the time of use is an operation that is very difficult to carry out under rigorous aseptic conditions, especially as the sterilization of the protective products is very tricky, because it must neither deteriorate the protective product itself (the products containing albuminoids coagulate with heat, for example) nor allow toxic compounds to exist in the sterilized stored protective product for the microorganisms.

The objective of the invention is therefore to create a vehicle for nonautonomous microorganisms of the animal kingdom stored alive, capable of being stored over prolonged periods ready to use; that is, containing the protective product from the time of formulation, and sterilized.

That is obtained with a vehicle for nonautonomous microorganisms of the animal kingdom, such as gametes or embryos, to be kept alive outside their natural environment with a view to human interventions, capable of being stored ready to use over prolonged periods, this vehicle being an aqueous medium comprising, especially besides nutrition agents, buffers and mineral salts, a protective product formed as support for embryonic growth by a living organism, and which in the state of the technique, is of animal origin and added immediately before use of the vehicle, characterized in that said protective product is a lecithin extracted from soy seeds, and introduced into the aqueous medium upon formulation of the vehicle.

It has been noted that, unpredictably, lecithin extracted from soy seeds, although derived from the plant kingdom and forming part of the support for development of the embryo of a seed, displays a protective power comparable to that of the usual protectors with regard to cell membranes belonging to the animal kingdom, without sharing their fragility in the face of the sterilization processes and poor storage over time, which made it possible to incorporate it in the vehicle during formulation of the latter, and to sterilize the vehicle with all its components. Under these conditions, the vehicle could be stored at refrigerator temperature (typically 4 °C) for prolonged periods, attaining at the least six months, without detectable deterioration.

Preferably, for cryogenic storage of nonautonomous microorganisms, the vehicle will contain a polyol at effective dose that is capable of inhibiting the formation of ice crystals, typically glycerol. The use of such a polyol, standard in cryogenic storage, proved to be compatible with the vehicle of the invention.

Also preferably, the vehicle is formulated with a reduced amount of water, and it is diluted with sterile water for use. Thus, the volume of the vehicle is reduced during storage between its formulation and its use; the provision of sterile water and the dilution of the concentrated vehicle without affecting the sterility of the final vehicle do not present any particular difficulty comparable with the prior practice of addition of the protective product immediately before use.

In a preferred arrangement, a vehicle especially designed for bovine sperm comprises in the concentrated state for 200 mL of water:

Trimethylol methylamine	3.4 g to 4.2 g
Trisodium citrate dihydrate	13.7 g to 16.75 g
Potassium chloride	0.55 g to 0.67 g
Fructose	1.65 g to 2.0 g
Glucose	0.68 g to 0.84 g
Lactose	0.41 g to 0.50 g
Calcium lactate	0.09 g to 0.11 g
Glycine	5.15 g to 6.25 g
Glycerol	64 mL to 78 mL
Soy lecithin	6.75 g to 8.25 g

this vehicle being diluted for use with 750 mL to 900 mL of water.

Secondary characteristics and advantages of the invention will moreover emerge from the description that follows by way of example.

Example 1 – Vehicle for bovine sperm

A vehicle is formulated by dissolving or dispersing in 200 mL of sterile water 3.809 g of trimethylol methylamine buffer (Tris buffer), 15.238 g of trisodium citrate dihydrate anticoagulant, 0.609 g of potassium chloride, of sugars, 1.828 g of fructose, 0.761 g of glucose and 0.457 g of lactose, 0.100 g of sodium lactate, 5.714 g of an amino acid, here glycine, 71 mL of glycerol and 7.5 g of soy lecithin. For use, the quantity of water will be brought to 1025 mL by addition of 825 mL of sterile water.

After formulation, the concentrated vehicle is sterilized in the usual way, put in a tight bottle and stored in a refrigerator at +4 °C.

Example 2 – Use of the vehicle

In the laboratory, bull sperm is frozen, each ejaculate being divided into two parts; one part was introduced into a vehicle in conformance with Example 1 after dilution, and the other part was introduced into a diluent with a formulation owned by the Applicant, including milk and egg yolk. In a standard way, to each part was added the usual antibiotics in equivalent proportions. After which, both parts were put separately into tubes called straws which were sealed and immersed in liquid nitrogen, according to a standard process.

Example 3 – Monitoring of the quality of storage of the spermatozoa in vitro

An equal number of straws belonging to both parts of the ejaculates from several bulls (to reduce the influence of the individual), and chosen at random, were removed from the liquid nitrogen and thawed for microscopic examination, while determining the percentages of motile and progressive spermatozoa. These percentages are revealed to be of the same order for the standard diluents and that according to the invention, with an advantage for the diluent according to the invention; but this advantage is not significant in terms of probability.

Example 4 – Monitoring by in vivo fertilization

Two lots of straws are chosen as with Example 3 that belong respectively to both parts, to fertilize in vitro oocytes sampled from cows and the number of oocytes fertilized is counted as well as the number of embryos that reached the blastocyst stage. There again the percentages of success proved to be approximately equal for both lots, with an advantage for the lot comprising the diluent of the invention. However, on account of the dispersion resulting from the diversity of oocytes, the advantage was not significant in terms of probability.

It will be observed that the preceding examples were designed to verify that the diluent of the invention was at least as effective as the standard diluents, and could stress a significant advantage of the invention, which is to operate routinely under sterile conditions. In fact, on the one hand, the laboratory tests may be carried out with more care and in a better defined environment than work of an industrial nature, and on the other hand, the risk of contaminations created by the standard processes practically do not appear in the early stages surrounding the fertilization.

In another aspect, it has been verified that the concentrations of vehicle components in a range of ±10% around values given in Example 1 do not appreciably degrade the effectiveness of the vehicles with respect to the spermatozoa. It will be observed that glycerol and lecithin contribute less than the other components to the biological compatibility of the vehicle for the nonautonomous microorganisms; glycerol being from this point of view approximately neutral

and acting only to prevent bursting of the membranes when frozen whereas lecithin appears as an external protector for microorganisms.

Moreover, trials with composition of media for storage of oocytes have been made with a view to fertilization "in vitro" and culture of embryos before implantation in the uterus of females, starting from the usual media compositions and adding the soy lecithin formulation to them in substitution for the usual protective products added immediately before use, with results from the point of view of maintaining the life of the nonautonomous microorganisms at least comparable to the results usually obtained, whereas the aseptic conditions of the processes were clearly improved.

Of course, the invention is not limited to the examples described, but embraces all variants of execution within the scope of the claims.

Claims

1. Vehicle for nonautonomous microorganisms of the animal kingdom, such as gametes or embryos, to be kept alive outside their natural environment with a view to human interventions, capable of being stored ready to use over prolonged periods, this vehicle being an aqueous medium comprising, especially beside nutrition agents, buffers and mineral salts, a protective product formed as support for embryonic growth by a living organism, and which in the state of the technique, is of animal origin and added immediately before use of the vehicle, characterized in that said protective product is a lecithin extracted from soy seeds, and introduced into the aqueous medium upon formulation of the vehicle.

2. Vehicle according to Claim 1 provided for cryogenic storage of nonautonomous microorganisms characterized in that at an effective dose it contains a polyol capable of inhibiting the formation of ice crystals.

3. Vehicle according to one of Claims 1 and 2 characterized in that for storage between formulation and use, the concentrated vehicle includes a partial amount of water, a complement of water to be added for use.

4. Vehicle according to Claim 3 specially designed for bovine sperm, characterized in that in the concentrated state it comprises 200 mL of water:

Trimethylol methylamine	3.4 g to 4.2 g
Trisodium citrate dihydrate	13.7 g to 16.75 g
Potassium chloride	0.55 g to 0.67 g
Fructose	1.65 g to 2.0 g
Glucose	0.68 g to 0.84 g
Lactose	0.41 g to 0.50 g
Calcium lactate	0.09 g to 0.11 g

Glycine	5.15 g to 6.25 g
Glycerol	64 mL to 78 mL
Soy lecithin	6.75 g to 8.25 g

this vehicle being diluted for use with 750 mL to 900 mL of water.

5. Vehicle according to Claim 4 characterized in that it contains approximately for 200 mL of water:

Trimethylol methylamine	3.809 g
Trisodium citrate dihydrate	15.238 g
Potassium chloride	0.609 g
Fructose	1.828 g
Glucose	0.761 g
Lactose	0.457 g
Calcium lactate	0.100 g
Glycine	5.714 g
Glycerol	71 mL
Soy lecithin	7.5 g

this vehicle being diluted for use with approximately 825 mL of water.

European Patent Office

Application Number EP 95 40 1170

EUROPEAN SEARCH REPORT

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.[6])
X	THE JOURNAL OF PROTOZOOLOGY, vol. 17, No. 2, May 1970, pages 151-152, D. COX "Prolonged survival of Tetrahymena at 0.5°C in Citrated, Lecithinized, defined media." *abstract*	1-5	C12N 5/06
X	TRANSACTIONS OF THE AMERICAN FISHERIES SOCIETY, vol. 112, 1983, pages 86-94, J.H. KERBY "Cryogenic preservation of sperm from Striped Bass." *the entire document*	1-5	
X	GAMETE RESEARCH, vol. 17, 1987, pages 355-373, A.M. SIMPSON ET AL. "Susceptibility of epididymal Boar sperm to cold shock and protective action of Phosphatidylcholine." *the entire document*	1-5	
A	EP.A.0 521 674 (T. DOMINKO) January 7, 1993		TECHNICAL FIELDS SEARCHED (Int. Cl.[6]) C12N C12Q
A	EP.A.0 559 307 (W.R. GRACE & CO. -CONN) September 8, 1993		
A	FR.A.2 350 395 (MC DONNELL DOUGLAS CO.) December 2, 1997		

The present search report has been drawn up for all claims.

Place of search	Date of completion of the search	Examiner
The Hague	July 18, 1995	Cartagena y Abella, P.

CATEGORY OF CITED DOCUMENTS

X: Particularly relevant if taken alone.
Y: Particularly relevant if combined with another document of the same category.
A: Technological background.
O: Non-written disclosure.
P: Intermediate document.

T: Theory or principle underlying the invention.
E: Earlier patent document, but published on, or after the filing date.
D: Document cited in the application.
L: Document cited for other reasons.

&: Member of the same patent family, corresponding document.

United States Patent Office 3,444,039
Patented May 13, 1969

3,444,039

FREEZABLE LIVE CELL DILUENT AND PROCESS

Ambrose Harry Jesudasan Rajamannan, Anoka, Minn. (103 Landmark Drive, Eastern Heights, Ithaca, N.Y. 14850)

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ABSTRACT OF THE DISCLOSURE

This disclosure provides new and freezable diluent preservative material for live cells. The preservative material comprises dry and water soluble preparations of buffered polysaccharides, for example derivatives of partially hydrolyzed starches and essentially such water soluble buffered polysaccharides with or without modification, as including a water soluble extract of dried egg yolk solids therewith.

This invention relates to new and useful improvements in the art and method of preservation and freezing of live mammalian cell materials with partially hydrolyzed starches and essentially polysaccharides, and the products obtained thereby. More particularly, this new disclosure to the art concerns the provision of improved and more economical extender and preservative diluent material as freezable non-growth live cell preservative diluent, compounded in the critical relationship of an acidic solution of a mixture of predominantly carbohydrate polysaccharides and a buffering agent conditioning the mixture to a critical pH range of 6 to 7.5, and which contains over 80% carbohydrate polysaccharides exclusive of mono and disaccharides and inclusive of at least 30% mixture of Tri to Hepta saccharides, said mixture in solution, having an osmotic pressure in the critical range of 225 to 350 milliosmoles; including the combination therewith of a liquid extract from dry solids of egg yolk for mixing with such live cell and cell tissue matter as semen, germ plasma, bone marrow cells, muscle and artery tissue cells, and the like cell matters, which live cell matters are killed or damaged beyond practical use in the presence of the partially hydrolyzed vegetable starches constituting the high quantity polysaccharides when utilized without such conditioning, the method of preparing the said critically buffered preservative diluent material, and the combination therewith of such live cell materials.

The art has particularly been faced with the problem of cell damage in preservation and freezing in sugar, alcohol and other preservatives heretofore disclosed and known to the art. One of such prior disclosures is that found in Canadian Patent No. 682,906 issued Mar. 24, 1964. In such disclosure the problem of metabolism has led to the use of expensive and costly mediums which include primarily polyhydric alcohols of 4 to 7 carbon atoms, as sorbitol, mannitol, inositol, erythritol and the like. The prior art also includes the use of such materials as glucose, fructose, adonitol, and the like. In general, the fault with such materials is not only the cost factor, as there has remained the major problem of a decrease in healthy cells recovered after freezing. Even in the most expensive preservatives perfected, a high percentage of the cells recovered after freezing are injured and defective. It will thus be recognized that an alternative and new preservative diluent formulation of greater economy in manufacture and at the same time providing an improved recovery of live healthy cells would be of new advantage and is of need in the art.

Accordingly, it is the principle purpose of this disclosure to provide the art with improved preservative diluents which are economical to produce and by their

use provides an improvement in the recovery of healthy live mammalian cells, including live tissue cell matter, after preservation by freezing in admixture with the diluents, as herein provided.

Another object of this disclosure is to provide a method of preparing the preservative diluents herein provided from a low cost mixture of naturally acidic carbohydrate polysaccharide material, of predominantly polysaccharides obtained by partial hydrolysis of naturally occurring vegetable starches and obtaining herewith an improved economical preservative diluent for live mammalian cells and which affords an improvement in recovery of healthy live sells after freezing.

In general, it may be stated that to obtain the new and useful objects and advantages of the method and preservative diluents herein provided comprises the preparation of a very mildly alkaline or buffered solution of an essentially normally acidic mixture of said carbohydrate polysaccharides material, as a mixture of partially converted hydrolysis products of naturally occurring starches of the vegetable kingdom, in the critical pH range of 6 to 7.5 and with an osmotic pressure between the critical range of 225 to 350 milliosmoles, in combination with an extract of a liquid fraction from spray dried egg yolk solids, and prepared in a temperature range of 2° C. to 37° C. This diluent and preservative is thereafter combined and used with the indicated live cell materials in a conventional manner. The preferred use of the prepared diluent, with added live cell matter, is in the freshly prepared freezable liquid form. However, the liquid portion of the prepared diluent may be evaporated, as by spray drying, or freeze drying, and the diluent solids later dissolved in water ready for extender and preservative use.

For the purpose of exemplification, the said mixtures of carbohydrate polysaccharides composition, in prepared form as preservative diluents, will be hereinafter illustrated by their advantageous use in the field of preserving animal bovine semen for insemination.

To the accomplishment of the foregoing and related ends, this improvement in the art therefore comprises the features hereinafter more fully described and particularly pointed out in the claims, the following description setting forth in detail certain illustrative embodiments provided in this disclosure, these being indicative, however, of the various ways in which the principles of this disclosure may be employed.

To illustrate by specific examples the following are provided:

EXAMPLE 1

Sodium citrate	grams	54
Water	cc.	2,000
Spray dried egg yolk	grams	100
Partially hydrolized starch solids (grams per 1,000 cc. liquid extract)		5

The basic buffer solution of sodium citrate and water was prepared at slightly below normal room temperature. Then, the egg yolk material was dispersed in the prepared buffer solution. Upon mixing, a coagulum forms which is separable from the clear liquid by such conventional means as centrifuging, filtering or decanting. In this instance the mixture was allowed to stand until it had separated into two parts, (1) an upper coagulum fraction and (2) a lower clear liquid fraction. The lower clear fraction was extracted and to each 1,000 cc. clear fraction of extract was added 5 grams of partially hydrolyzed starch in the form of corn syrup solids and which are essentially over 80% polysaccharides. This constitutes a liquid diluent which is ready for use in a conventional manner, or in preparation for a live cell freezing process. If it is desired to freeze the solution of preservative dilu-

ent and live cell matter mixed therein, there is added about 5% to 10% glycerol by volume. The glycerol may be added before or after the mixture of live cell matter with the prepared liquid preservative diluent.

EXAMPLE 2

Alkaline citrate salt	grams	27
Water	cc	1,000
Egg yolk solids	grams	50
Corn syrup solids	do	5

The above components may be merely added together and mixed. However, it is preferred to prepare the base buffering solution by mixing the alkaline citrate salt, as sodium or potassium citrate in the water and then add corn syrup solids and spray dried egg yolk solids followed by thorough mixing. After mixing, the mixture is allowed to stand until it has gradually separated into two fractions, as above indicated. The lower clear fraction is extracted and ready for use, with the desired amount of live cells added. The addition of the cell matter to an extender liquid, in any amount, for practical use is optional and known to the art. If it is desired to freeze the mixture of preservative diluent and cells, about 5% to 10% glycerol, by volume, is added.

The following illustration will show how some modification of the solids portion may be made:

EXAMPLE 3

Buffering agent to pH 6 to 7.5 and osmotic pressure	milliosmoles	250–300
Water	cc	1,000
Egg yolk solids	gms	25–100
Carbohydrate polysaccharides (defined) (preferably 2 to 15 gms. if no complementary sugar or alcohol is added)	gms	.5–15
Fructose and/or glucose	gms	0–2

The above components were mixed as in Example 2 including separation of the desired liquid extract. The extract may also be prepared in the manner of Example 1. The buffering agent, as utilized herein, may be any suitable salt or alkaline agent, or mixtures of the same. For example, such agents as citrates, acetates, phosphates, carbonates, and the like may be used to neutralize the otherwise acid sperm killing nature of the saccharides and effect the pH control, as described. The carbohydrate saccharides are a mixture of a minimum of mono and disaccharides with a maximum of tri, tetra, penta, hexa, hepta and higher polysaccharides. The products utilized herein are obtained by partial acid and/or partial enzymic hydrolysis of naturally occurring starches as obtained from corn, maize, potatoes, wheat, rice, tapioca, oats, and other plant and vegetable matter of the vegetable kingdom. The hydrolysis products are a partial conversion between 10% to 50% dextrose equivalent. This mixture of carbohydrate polysaccharides cannot be used without a soluble neutralizing or buffering agent as alkali metal citrates, phosphates, acetates or carbonates and mixtures of the same, or the like. An economical source of the carbohydrate saccharides is preferably those obtained by the partial hydrolysis of naturally occurring starches as found in maize, corn, molasses and the like syrup materials. The conventional sugar material is used to compliment the saccharides. However, such sugars and alcohols as heretofore known to the art are preferably entirely eliminated.

EXAMPLE 4

The process of Example 1 using sorghum syrup in the formula proportion of Example 2.

In the above formulations a faster wetting of mixtures with the dried egg yolk solids was noticed when the said partially hydrolyzed starch materials were added, in the stage of effecting extraction of the dried egg yolk solids. This did appear to measurably reduce the mixture time. In view of the relative similarity in production of partial hydrolysis of the vegetable starches, it is not believed to be necessary to further describe other formula and similar naturally occurring starches in the form of use as partially hydrolyzed starches and polysaccharides as herein described.

In the illustrative specific examples the relative percentages of solids are on the order of about 33% buffering agent, about 60% dried egg solids and about 6% saccharides solids. In general, the relative solids may vary on the order of 87% to 96% egg yolk solids to about 3.8% to about 13% carbohydrate polysaccharides, with the buffering agent present in an amount sufficient to effect a pH in the critical range value of 6 to 7.5 and the solution in water having an osmotic pressure in the critical range of 225 to 350 milliosmoles.

The preparation of preservative and extender mediums in combination with live cell materials is well known to the art and as described in the above mentioned patent. The particulars therefore, in preparing live cell mixtures of the herein described preservative diluent and extender are optional with regard to concentrations of cell matter and diluent, as is known to the particular field of use in preservation and extending of live mammalian cell matter. For the above tests the dilution was 1 part semen to 100 parts preservative and extender diluent, as is commonly used for insemination of bull semen. The application for insemination is otherwise of the same order as known to the art.

In tests of bovine semene in admixture with the herein described preservative diluent material, compared to like semen tests of a standard yolk citrate medium and another called "Minnesota Go," a popular sugar alcohol extender, the following results were obtained:

1. With Low Quality Semen Applicant's (Bovine)

	1. Std. Yolk Citrate	2. "Minn. Go"	3. Improved Diluent of Example 1
Storage Ambient Temp	1 day Viability	2 day Viability	2 day Viability.
Plus 5° C	3 day Viability	5 day Viability	7 day Viability.
2. High Quality Semen (Bovine)			
Storage Ambient Temp	1 to 2 days	3 days	3 days.
Plus 5° C	5 days	7 days	7 days.
POST FREEZING SURVIVAL			
3. High Quality Semen (Bovine)			
Before Freezing	60% Motility	60% Motility	60% Motility.
Recovery After Freezing to Liquid Nitrogen Temp.	30% Motility	40% Motility	55% Motility.*
4. Low Quality Semen (Bovine)			
Before Freezing	40% Motility	40% Motility	40% Motility.
Recovery After Freezing	5% Motility	20% Motility	35% Motility.*

*Progressive type of motility showing no sperm cell damage and an amazing increase in survival capacity.

The proof of the advantages which are gained by the herein described new and useful preservative diluent is illustrated from the post freezing survival pattern in low quality semen. Bulls of high performance (normally associated with low quality semen) can be utilized to a more beneficial extent since the freeze kill and cell damage is minimized. That is, the freeze kill has been reduced and a higher percentage of live cells are recovered, enabling a high quality semen producing bull to be used on a larger scale. Under the frozen semen program, the low quality semen producing bulls, many valued at $10,000 or more, would otherwise be discarded, with heretofore known and utilized semen preservative medium, despite high pedigree and performance. However, by use of my new preservative diluent compositions, such bulls can be saved and their semen used more productively.

In similar experimental tests with like diluted mixtures of frozen live cell matter, as the semen of cocks, studs, rabbits, pigs, dogs, rams and goats, with the carbohydrate saccharide medium herein provided, the recovered unfrozen mixtures, under microscope analysis, showed a comparable progressive type of motility with high activity and no appreciable cell damage.

Illustrative of a different application, a small sample of blood was taken and mixed with the prepared carbohydrate polysaccharide composition, minus the egg yolk extract, in the proportion of about 1 part blood to 2 parts of said composition. This mixture was frozen under liquid nitrogen temperature condition. Upon microscopic comparison of the unfrozen blood cells, with a new fresh sample from the same blood source, no visible cell damage was apparent. Inasmuch as the carbohydrate polysaccharide material may be a normal human food material, in its liquid state it may also be used as a preservative in the field of frozen blood for transfusion and frozen preservation of muscle, ligament and tissue cell matter, under suitable hygienic conditions and with or without a suitable antibiotic, as known to the art. To those skilled in the art, the equivalent and similarity of the preservative diluent for use with mammalian cell material will be recognized.

From the above description and subject matter of disclosure it will be apparent that some modifications as herein set forth may be made without departing from the spirit and scope thereof, as embodied in the terms of the following claims.

I claim:

1. In the process of preparing a freezable diluent for live cells the steps of preparing a water solution with an alkaline buffering agent, mixing dried egg yolk in the solution, effecting a separation of a solids portion and a clear liquid portion of said mixture, removing the clear liquid portion from said solids portion, retaining the clear liquid fraction and adding thereto the partially hydrolyzed products of naturally occurring starches consisting principally of over 80% polysaccharides of which over 30% consist of tri, tetra, penta, hexa and hepta saccharides.

2. The method of preparing a preservative diluent for live mammalian cells comprising preparing a water solution of dried egg yolk solids and a buffering agent, extracting the liquid from the solids, and adding from about 0.5 part to 15 parts water soluble carbohydrate polysaccharide material per about each 1000 cc. of water, the addition of said buffering agent in relationship to said polysaccharide material being added in an amount to effect a pH value of said solution in the range of 6 to 7.5 and an osmotic pressure of 225 to 350 milliosmoles.

3. The method of claim 2 wherein the proportion of said polysaccharide material is added between the range of 0.5 to 15 parts with a complementary sugar.

4. The method of preparing a freezable preservative diluent for live cells comprising preparing a water solution of a buffering material, dried egg yolk solids and carbohydrate polysaccharide material, separating the solids from the liquid and retaining the separated liquid as the preservative medium having a pH value in the order of 6 to 7.5 and an osmotic pressure of 225 to 350 milliosmoles.

5. The product of claim 4 including live cells contained in a frozen state with said medium.

6. A freezable preservative and extender diluent for live cells comprising a mixture of a combination of water soluble carbohydrate polysaccharides and a buffering agent therefor, said mixture in a water solution having a pH value of 6 to 7.5 and an osmotic pressure of 225 to 350 milliosmoles.

7. The product of claim 6 containing a proteinaceous egg yolks.

8. The product of claim 6 including in combination therewith live mammalian cells in a frozen state.

9. A freezable preservative and extender diluent for live bovine sperm cells comprising a mixture of a water soluble extract from dried egg yolks, water soluble carbohydrate polysaccharide material, an alkaline buffering agent for said polysaccharide material, said mixture in a water solution having a pH value in the range of 6 to 7.5 and an osmotic pressure in the range of 225 to 350 milliosmoles.

10. The product of claim 9 wherein the said carbohydrate polysaccharide material is selected from the group consisting of the partially hydrolyzed end products of natural starches of the vegetable kingdom.

11. The product of claim 9 wherein the carbohydrate polysaccharides material is corn syrup material.

12. The product of claim 9 wherein the carbohydrate polysaccharide material is the partially hydrolyzed end product of corn starch.

13. The product of claim 12 including live mammalian cells contained with the said diluent in a frozen state.

14. An improved freezable preservative and extender diluent for live mammalian cells comprising a combination in the proportion of a water extract of about 25 to 100 parts dried egg yolk solids, about 0.5 to 15 parts water soluble carbohydrate polysaccharide material, and a buffering agent for said polysaccharide material providing a pH value in the range of 6 to 7.5 in a water solution.

15. The product of claim 14 including live mammalian cells.

16. The product of claim 14 wherein the carbohydrate polysaccharide material is selected from the group consisting of partially hydrolyzed natural starches obtained from the vegetable kingdom.

17. The product of claim 14 wherein the carbohydrate polysaccharide material is present in a proportion of about 0.5 to 15 parts in combination with up to about 2 parts complementary sugar.

18. A freezable preservative diluent for live cells containing a polysaccharide material comprising a partially hydrolyzed vegetable starch with a 10–50% dextrose equivalent and a buffering agent, said preservative in a water solution having a pH value of 6 to 7.5 and an osmotic pressure of 225 to 350 milliosmoles.

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F. CACCIAPAGLIA, JR., *Primary Examiner.*

U.S. Cl. X.R.

62—1; 195—1.7

US006130034A

United States Patent [19]

Aitken

[11] Patent Number: 6,130,034

[45] Date of Patent: Oct. 10, 2000

[54] **USE OF CYB MEDIUM FOR THE TRANSPORTATION AND STORAGE OF SPERM**

[75] Inventor: **Robert John Aitken**, Edinburgh, United Kingdom

[73] Assignees: **Medical Research Council**, Edinburgh, United Kingdom; **Applied Research Systems ARS Holding N.V.**, Curaçao, Netherlands Antilles

[21] Appl. No.: **09/074,465**

[22] Filed: **May 8, 1998**

Related U.S. Application Data

[63] Continuation of application No. PCT/GB95/02635, Nov. 9, 1995.

[51] **Int. Cl.**[7] **A01N 1/02**; A61B 17/43

[52] **U.S. Cl.** **435/2**; 600/33; 600/35

[58] **Field of Search** 435/2; 600/33, 600/35

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Primary Examiner—Sandra E. Saucier

Attorney, Agent, or Firm—Sterne, Kessler, Goldstein & Fox P.L.L.C.

[57] **ABSTRACT**

The use of CYB medium for the storage and/or transportation of semen at ambient temperature.

6 Claims, No Drawings

USE OF CYB MEDIUM FOR THE TRANSPORTATION AND STORAGE OF SPERM

This is a continuation of the International application PCT/GB95/02635 filed Nov. 9, 1995.

The present invention relates to a novel method for storing and transporting sperm which ensures its viability.

Diagnosis of male infertility has become increasingly more complex over recent years in view of the increasing sophistication of andrological techniques. This has led to the creation of specialised andrology centres which possess the necessary expertise in techniques such as computerised image analysis, cell biology and biochemistry, and can thus offer the most up to date diagnostic techniques.

However, a limitation on the growth of such specialised centres is that patients have to attend the centre in order to provide a semen sample immediately prior to analysis. Fresh samples of semen are required since semen loses viability and functional competence when left in the presence of seminal plasma for any length of time, usually after 1 hour.

There thus exists a need to provide a method for storing/preserving semen samples at ambient temperature such that they can be sent to andrological centres for diagnostic testing, removing the need for patients to attend personally.

The present inventors have now found that semen can be stored at ambient temperature in a particular medium known as an egg yolk buffer.

Thus, in a first aspect, the present invention provides a medium for storage and/or transportation of semen at ambient temperature which comprises CYB medium.

CYB medium is a known cryoprotectant medium first described by Weidel, L. and Prins, G. S., *J. Androl.* 8: 41–47 (1987). It consists of the following components:

40% TES/TRIS buffer

30% sodium citrate/fructose solution

20% fresh egg yolk

1% pen-strep solution (10,000 IU).

TES=N'-Tris (hydroxymethyl)methyl-2-aminoethane sulphonic acid.

TRIS=Hydroxymethylaminomethane.

The TES/TRIS buffer contains 8.66 g TES and 2.06 g TRIS per 200 ml of water.

The sodium citrate/fructose solution contains 5.88 g sodium citrate and 4.0 g fructose per 200 ml of water.

The constituents of the CYB medium are mixed together and centrifuged at 600 g for 2x10 mins to remove particulate matter. The pH is adjusted to 7.4.

In a preferred embodiment, the medium also includes an antioxidant. Suitable antioxidants include α-tocopherol (vitamin E), catalase, glutathione and mannitol. It is believed that these components will act as free radical scavengers. A particularly preferred antioxidant is α-tocopherol, present at a concentration of 1 mM.

In a second aspect, the invention provides CYB medium for use in the storage/transportation of semen at ambient temperature.

In a third aspect, the invention provides the use of CYP medium for the storage and/or transportation of semen at ambient temperature.

In a fourth aspect, the invention provides a method for storing and/or transporting semen at ambient temperature which comprises the step of bringing the semen into contact with CYB medium. In this aspect of the invention, the semen is diluted approximately 1:1 with the CYB medium.

In preferred embodiments of all of the above-noted aspects, the semen is human semen.

Preferred features of each aspect of the invention are as defined for each other aspect, mutatis mutandis.

The invention will now be described by way of the following examples which should not be construed as in any way limiting the invention.

EXAMPLE 1

(A) Methods

(i) Sample Density Protocol

10 μl of sperm suspension was added to 190 μl of sperm diluting fluid (SDF; see below), mixed well, and both sides of the counting chamber were filled (improved Neubauer ruling).

This was then left to settle.

The number of sperm in 5 large squares running diagonally across the slide was counted, each large square having 16 smaller squares.

When sperm heads lay on the border, only those that lay on the top and left side border were included.

The number of sperm in 5 large squares=million sperm per ml (10^6/ml).

Sperm Diluting Fluid (SDF)

$NaHCO_3$	50 g
Formalin	10 ml
Made up to 1 liter in distilled water.	

(ii) Scoring Motility

10 μl of sperm suspension is placed on a slide and covered with a 24x24 mm coverslip. Using an eyepiece graticule with a square grid, an area is defined and motile sperm within that area are counted. Non-motile sperm within the area are counted. The field is moved and repeat process until at least 100 sperm have been counted. % motility (motile sperm as % of total sperm counted) is calculated.

(iii) Percoll Prepared Sperm

3 ml of 100% prepared percoll solution (see below) was placed in the bottom of a test tube. 3 ml of 50% prepared percoll solution (see below) was carefully layered on top of the 100% percoll (50% percoll was layered 1 ml at a time using a 1 ml pipette).

2 ml of semen sample was layered on top of the gradient column. This was then centrifuged at 1900 rpm for 20 mins (500 g).

Seminal plasma was removed from the top of the column (saved frozen).

The sperm was separated in bands (50% from the middle of the gradient at the 50/100 interface, and 100% from the bottom of the tube). This was resuspended in 7–10 ml of BWW (see below) and centrifuged at 1900 rpm for 5 mins.

The supernatant was removed and the sperm pellets resuspended in a known volume of BWW (500 ml -1 ml depending on recovery of sperm pellet).

Density was recorded and the sperm concentration was adjusted to $20x10^6$/ml.

Discontinous Percoll Gradients

100% Percoll Solution—100 ml

10 ml of 10xEarle's Balanced Salts Solution FLOW LABS, IRVINE, SCOTLAND

90 ml of percoll PHARMACIA LKB BIOTECHNOLOGY AB, UPPSALA, SWEDEN

6 ml of albuminar 5% ARMOUR PHARMACEUTICAL COMPANY, EASTBOURNE, ENGLAND

3 mg of sodium pyruvate

0.37 ml of sodium lactate

200 mg of sodium hydrogen carbonate ($NaHCO_3$)

50% Percoll Solution
100% percoll solution 1:1 diluted with BWW.
BWW Preparation
BWW Stock—Made up in 1 liter of distilled water.

NaCl	5.54 g
KCl	0.356 g
$CaCl_2$ (dihydrate)	0.250 g
KH_2PO_4	0.162 g
$MgSO_4 \cdot 7H_2O$	0.294 g

BWW—200 ml

$NaHCO_3$	420 mg
Glucose	200 mg
Sodium pyruvate	6 mg
Albuminar 5% (= 0.3% Final)	12 ml
Sodium lactate	0.74 ml
Penicillin/streptomycin GIBCO	2.0 ml
Hepes buffer FLOW LABORATORY, IRVINE, SCOTLAND	4.0 ml
BWW stock	181 ml

(B) Sample Treatment

(i) Patient semen samples were obtained in 30 ml sterile plastic sample containers. A 30 minute liquification period was allowed prior to recording sample volume, density and round cell count.

(ii) The samples were then split into two aliquots and treated as follows:

One aliquot was prepared on percoll gradients as detailed above.

The remaining aliquot was mixed with a fixed volume (4.0 ml) of CYB medium, thereby providing at least a 1:1 dilution for most semen samples. Sample motility was checked again before packaging in an insulated polystyrene box for despatch by a courier service over a period of 24 hours.

Aliquots prepared on Percoll gradients were analysed as follows:

(a) Acrosome Reaction Assay

Sperm samples were prepared according to the 50%/100% percoll gradient protocol, adjusting the sperm density to 20×10^6/ml.

200 μl of sperm was added to an equivalent volume of A23187 free acid to give a final concentration of 1.25 μM or 2.5 μM A23187.

The mixture was incubated at 37° C. for 3 hours.

The mixture was washed at 500 g for 5 minutes and the supernatant removed and resuspended in BWW at 20×10^6/ml.

Motility of the sample was recorded.

50 μl of sperm suspension was added to 500 μl of Hypo-osmotic Swelling Medium and incubated for 1 hour at 37° C.

The suspension was centrifuged for 5 minutes at 500 g, the supernatant discarded and the pellet resuspended in 50 μl of ice cold methanol, i.e. final sperm concentration 20×10^6/ml.

10 μl was put onto each spot of a Hendley slide and was allowed to air dry.

This was then overlayed with Lectin-Fluorescein Isothiocyanate (2 mg/ml in PBS) and incubated for 15 minutes in the dark.

Excess lectin was then washed with PBS.

A drop of Citifluor was put on each spot of the Hendley slide and the slide was then covered with a coverslip for examination under a fluorescence microscope.

Hypo-osmotic Swelling Medium

7.35 g sodium citrate
13.51 g fructose
1 liter of distilled water.

(b) Sperm Penetration Assay

Sperm select 1:1 was diluted with BWW.

The diluted sperm select was then loaded into flat capillary tubes (200 μm).

One end of the tube was capped with Critoseal (Mackay and Lynn, Edinburgh).

The open end of the sperm select capillary tubes was placed into the semen sample (50 μl).

This was then incubated at 37° C. for 30 min at an angle of 20° (approx).

The sperm select and Penetrak tubes were removed from the sperm, placed on a marked microscope tube and the number of sperm present at 1, 3 and 4.5 cm from the open end of the tube was counted (counting the number of sperm in 2 fields—using a ×40 objective).

Aliquots Sent by Courier

On arrival of the samples, the samples were analysed for motility loss before percoll preparation and diagnostic assays were repeated to determine if the transport diluent was capable of sustaining these important diagnostic parameters in the "field" situation. Experimental variation is reduced by the use of strict correlated protocols and by employing the same technician in the assessment of subjective assays such as the acrosome reaction test.

Results

(a) Sperm Motility

21 semen samples chosen from a random selection of donors were investigated. Motility before and after shipment in CYB medium was measured.

TABLE 1

Sperm Progressive Motility (WHO: a + b) in % motile on n = 21 donors	
Semen t = 0	Semen/CYB t = 24
8	24
11	14
17	16
22	20
26	46
30	65
32	30
36	30
44	48
47	59
48	55
49	58
53	30
54	39
60	56
62	71
64	68
73	64
45	55
76	61
80	69
mean = 46	mean = 47
SD = 22	SD = 19

These results show that Sperm Progressive Motility at t=24 h in CYB is on average well related to the response at t=0.

(b) Acrosome Reaction Assay

For the same 21 samples of semen as noted above, the sperm percentage of viable cells was as follows:

TABLE 2

Ionophore A23817

Semen t = 3	semen/CYB t = 24
21	34
25	55
27	28
37	19
43	66
57	42
59	61
60	56
61	83
64	62
70	80
74	79
78	62
78	82
79	75
79	82
79	81
81	87
87	90
mean = 61	mean = 64
SD = 21	SD = 21

These data show that the % cells viable, following treatment with Ionophore A23187 at t=24 h in CYB, is, on average, well related to the response at t=3.

(c) Sperm Penetration Assay

Sperm penetration (at t=1.5 cm, 3 cm and 4.5 cm) at t=0 and after t=24 h in CYB was as follows:

TABLE 3

Sperm penetration on n = 20 donors

1.5 cm t = 0	3 cm t = 0	4.5 cm t = 0	1.5 cm t = 24	3 cm t = 24	4.5 cm t = 24
251	34	2	166	27	8
174	19	1	24	4	3
121	6	0	35	7	0
224	43	8	250	47	10
110	19	2	85	18	1
260	54	9	255	110	8
196	20	3	358	74	6
275	45	8	86	26	3
270	91	25	284	99	28
180	19	3	300	117	15
2	0	0	3	1	0
6	2	0	5	21	0
28	2	0	34	5	1
192	45	9	186	43	7
92	18	1	101	21	2
114	11	0	109	12	1
210	21	2	215	23	3
300	40	7	270	38	8
34	3	0	39	5	1
170	37	5	156	32	6
mean = 160	mean = 26	mean = 4	mean = 36	mean = 36	mean = 6
SD = 93	SD = 23	SD = 6	SD = 111	SD = 36	SD = 7

There was no statistically significant difference between the values measured at t=0 and measured at t=24 hr in CYB.

EXAMPLE 2

General Semen Analysis (Other than Motility) of Spermatozoa Incubated in CYB for 24 Hours

Semen liquefication was assessed as being either non-existent, moderate or normal. In samples examined, the presence of mucous threads, ie incomplete liquefication was also backed up by drawing the sample through a pipette to determine if the fluid flowed freely. After mixing 1:1 with CYB and incubation for 24 hours the assessment was repeated.

Sample Number	Semen t = 0	Semen/CYB t = 24 hr
1	Normal	Normal
2	Normal	Normal
3	Moderate	Moderate
4	Normal	Normal
5	None	Moderate

Clearly, incubation in CYB has no significant effect on this observed property of sperm.

Abnormal form analysis was performed on sperm in semen and mixed 1:1 with CYB at t=0 hours and t=24 hours. The cells were fixed in standard formalin solution before analysis, the cells being observed using a ×40 objective.

Sample Number	CYB t = 0	CYB t = 24	Semen t = 0	Semen t = 24	% diff t = 0	% diff t = 24
1	65	54	61	55	17	10
2	68	83	78	69	18	11
3	68	52	54	48	24	11
4	70	76	71	73	8	3
5	74	74	79	78	0	1

Clearly, incubation with CYB does not appear to alter sperm morphology. Any discrepancies can be assumed to be due to sampling error.

Measurement of Antisperm Antibodies in CYB

Samples were treated with the blood serum of patients previously known to have high antisperm antibody levels. Thus, antibody was transferred to the test samples, allowing a clear estimation of the effect of CYB on antibody detection after 24 hours.

The semen sample was allowed to liquefy for 30 minutes and was then prepared on mini percoll gradients by centrifugation at 600 g for 5 minutes.

The semen was then washed twice in 0.4% BSA in Earles culture medium by centrifugation at 200 g for 5 minutes. The semen was then resuspended in 500 μl of 0.4% BSA in Earles culture medium.

500 μl of donor semen was incubated with 500 μl of serum for 1 hour. After incubation, the sample was washed twice with 0.4% BSA in Earles culture medium and then resuspended with 1 ml of culture medium and then mixed 1:1 with CYB.

For the MAR test the washed sperm were reconstituted into their seminal plasma before the MAR scoring. This involved mixing the semen with O+ blood preparation and IgG anti-sera. The IBBA test is amore sensitive and specific assay for the detection of anti-sperm antibodies.

	MAR t = 0	MAR t = 24 CYB	IBBA t = 24 CYB
Control	Negative	Negative	IgA/IgG -ve
Stock IgG	100% positive	100% positive	IgA 50% +ve IgG 90% +ve

-continued

	MAR t = 0	MAR t = 24 CYB	IBBA t = 24 CYB
1 in 2 IgG	100% positive	100% positive	IgA 10% +ve IgG 90% +ve
1 in 10 IgG	100% positive	100% positive	IgA 10% +ve IgG 90% +ve

The IBBA results corresponded to the results gained by the serum samples in previous assays. Thus, the action of CYB does not appear to impair the detection of anti-sperm antibodies.

What is claimed is:

1. A method for the storage and/or transportation of semen at ambient temperature which comprises the step of bringing the semen into contact with a medium comprising CYB medium, wherein said ambient temperature does not reach a level required for cryopreservation of sperm, and wherein the time period for said storage and/or transportation exceeds 1 hour.

2. The method as claimed in claim 1, wherein the semen is diluted 1:1 with the medium.

3. The method as claimed in claim 1, wherein the semen is human semen.

4. The method as claimed in claim 1, wherein the medium in addition to the CYB medium further comprises an anti-oxidant in sufficient quantity to prevent loss of motility.

5. The method as claimed in claim 4, wherein the anti-oxidant is α-tocopherol, catalase, glutathione or mannitol.

6. The method as claimed in claim 5, wherein the anti-oxidant is α-tocopherol and is present at a concentration of 1 mM.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,130,034

DATED : October 10, 2000

INVENTORS : Aitken

It is certified that an error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below.

On the title page, [75] Inventor, please delete "Edinburgh, United Kingdom" and insert therefor --Newcastle, NSW, Australia--.

Signed and Sealed this

Twenty-second Day of May, 2001

Attest:

Nicholas P. Godici

NICHOLAS P. GODICI

Attesting Officer

Acting Director of the United States Patent and Trademark Office

US006140121A

United States Patent [19]

Ellington et al.

[11] Patent Number: 6,140,121

[45] Date of Patent: *Oct. 31, 2000

[54] **METHODS AND COMPOSITIONS TO IMPROVE GERM CELL AND EMBRYO SURVIVAL AND FUNCTION**

[75] Inventors: **Joanna E. Ellington**, Valleyford; **Sylvia Adams Oliver**, Spokane, both of Wash.

[73] Assignee: **Advanced Reproduction Technologies, Inc.**, Valleyford, Wash.

[*] Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

[21] Appl. No.: **08/733,227**

[22] Filed: **Oct. 17, 1996**

Related U.S. Application Data

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[51] **Int. Cl.**[7] **C12N 1/00**; A01N 63/00

[52] **U.S. Cl.** **435/374**; 435/366; 435/404

[58] **Field of Search** 435/374, 366, 435/404

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Primary Examiner—Jeffrey Stucker
Assistant Examiner—Brett Nelson
Attorney, Agent, or Firm—Seed Intellectual Property Law Group, PLLC

[57] **ABSTRACT**

Sperm, oocyte, and embryo survival and function is improved in vivo or in vitro by the use of a polysaccharide containing arabinose, galactose and/or hexuronic acid. In particular, a nonspermicidal lubricant containing such a polysaccharide (e.g., gum arabic, pectin, or galacturonic acid) increases the fertilization potential of the sperm during coitus, artificial insemination or sperm collection. Similarly, a freezing medium containing a polysaccharide containing arabinose, galactose and/or hexuronic acid enhances sperm, oocyte, or embryo viability.

16 Claims, 19 Drawing Sheets

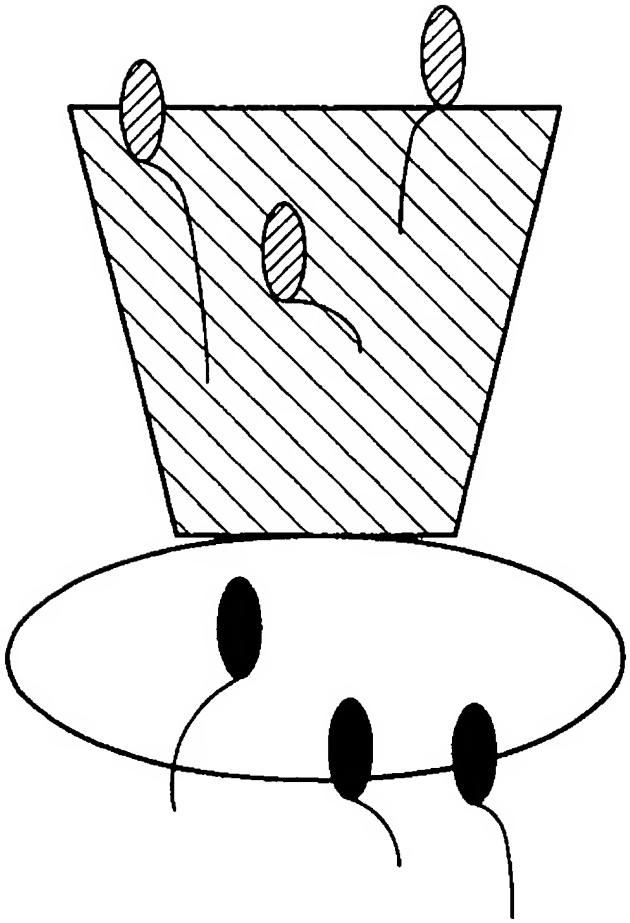


Fig. 1

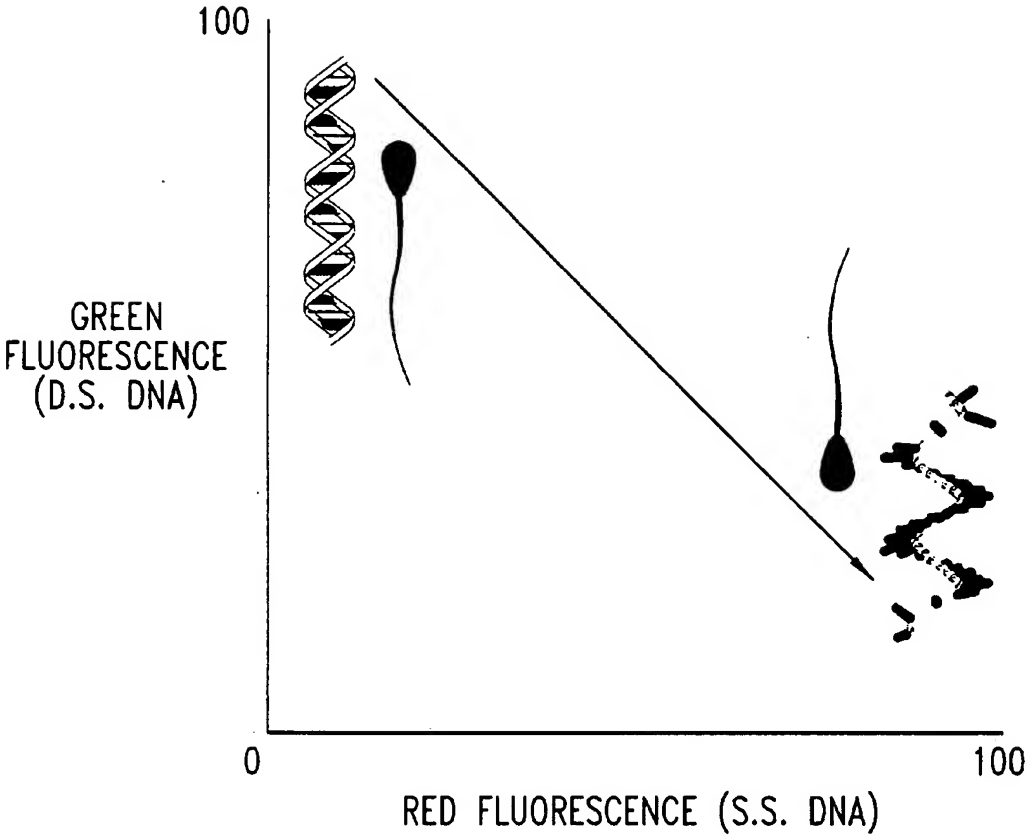
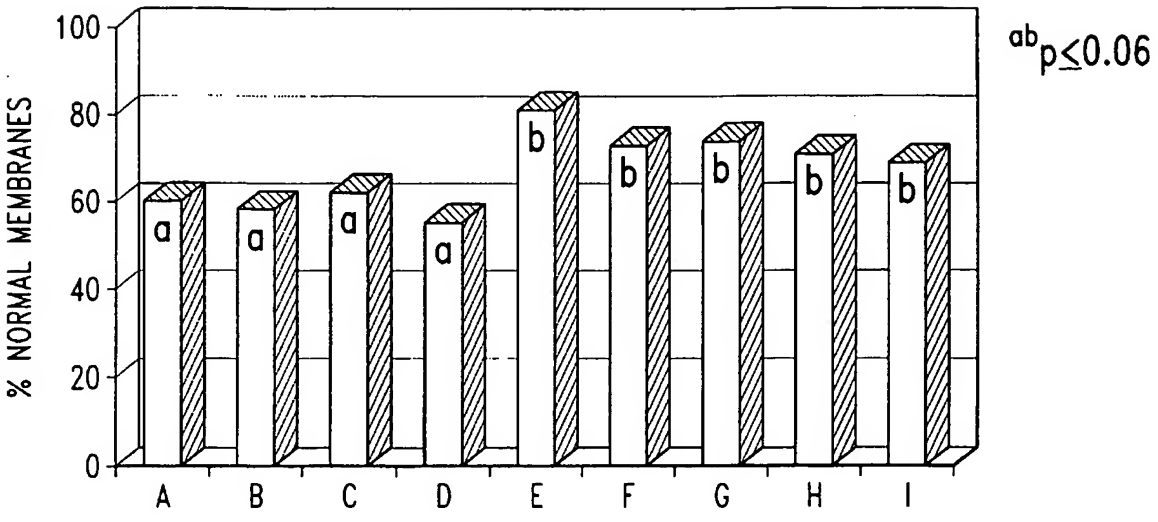
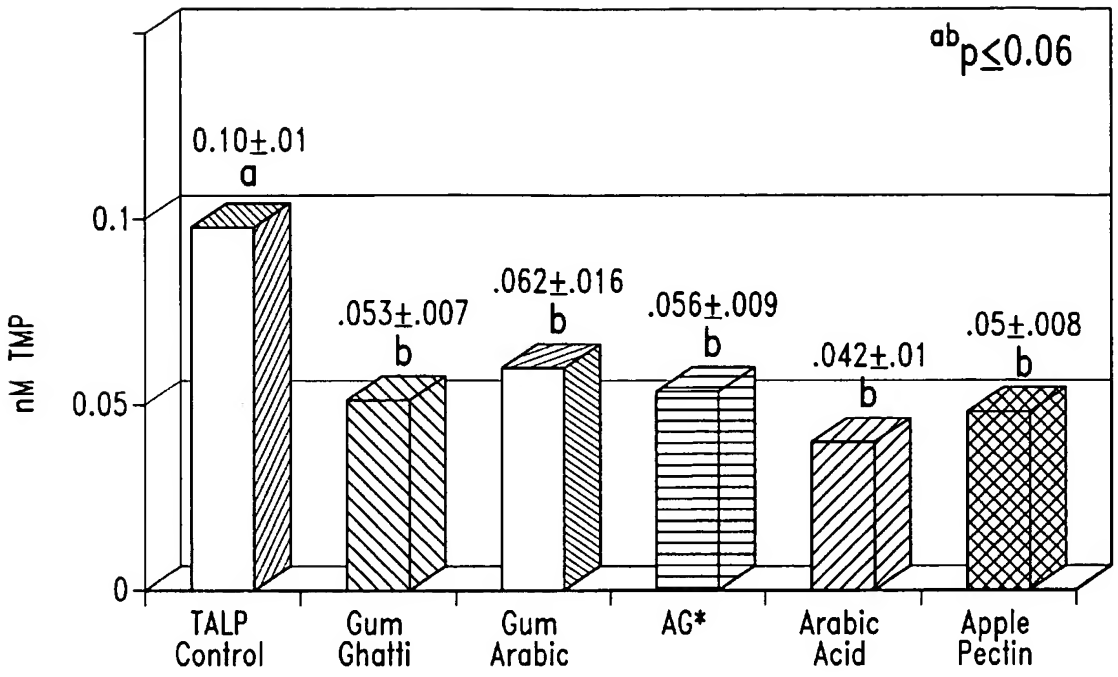


Fig. 2



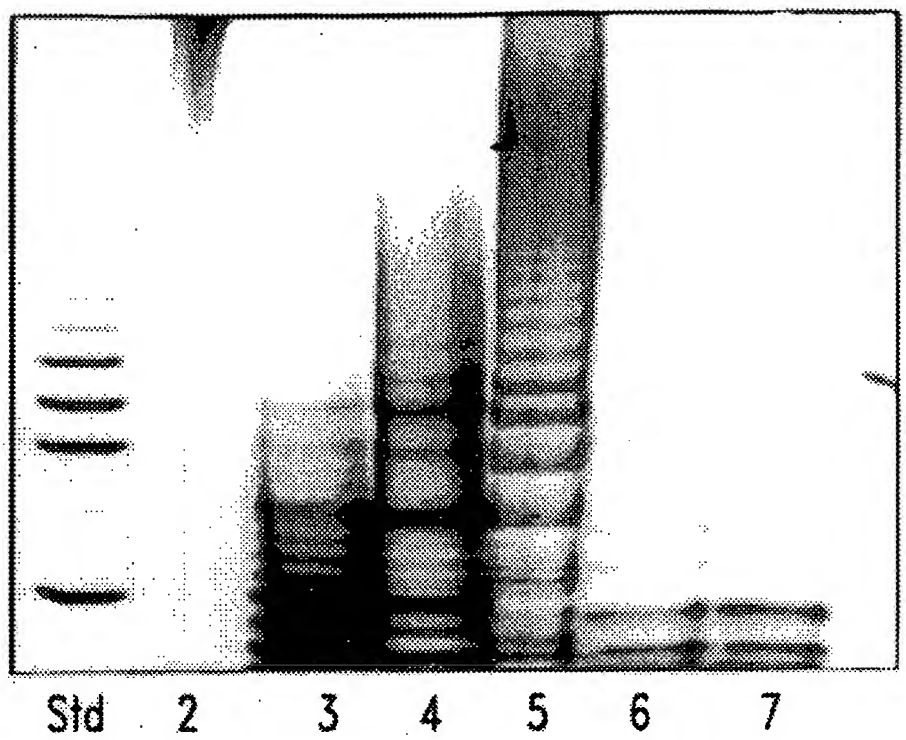
A: Control HTF
B: Arabinose (Ara)
C: Galactose (Gal)
D: Ara + Gal
E: Galacturonic Acid
F: Gum Guar
G: Galactopyranosylarabinose
H: Gum Karaya
I: Gum Locust Bean

Fig. 3



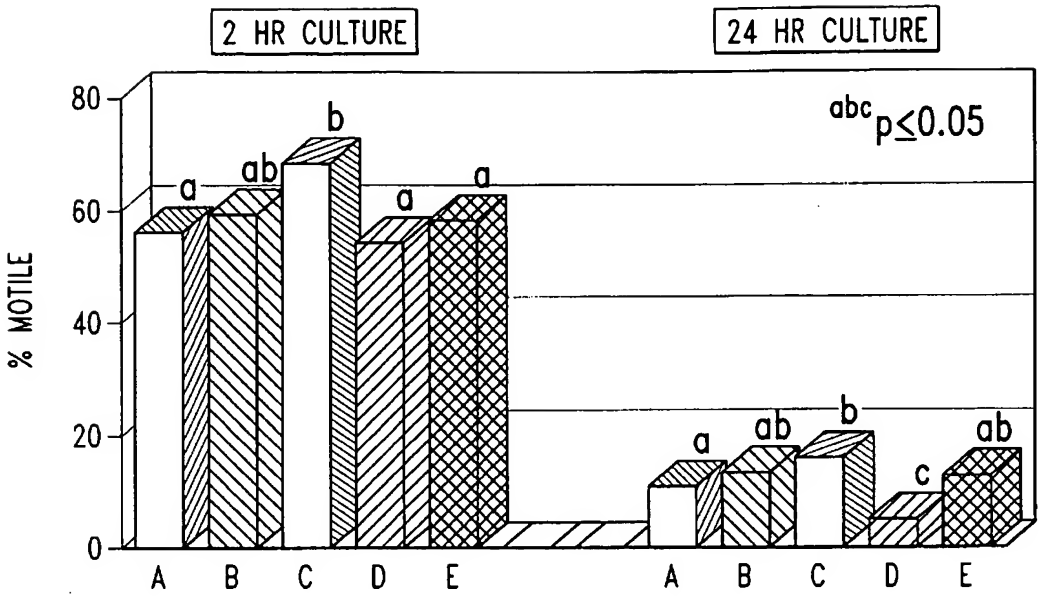
(*AG: arabinogalactan)

Fig. 4



Lane 2 Pectin uncut
Lane 3 Pectin cut with EndoA
Lane 4 Pectin cut with AF
Lane 5 Pectin cut with EndoPG
Lane 6 Pectin cut with all three enzymes together
Lane 7 Same as lane 6 but with twice as much loaded

Fig. 5



A: Control TALP
B: 0.05% Pectin in TALP
C: Pectin Fraction <100Kd
D: Pectin Fraction >100Kd. <0.2u
E: Pectin Fraction >0.2u

Fig. 6

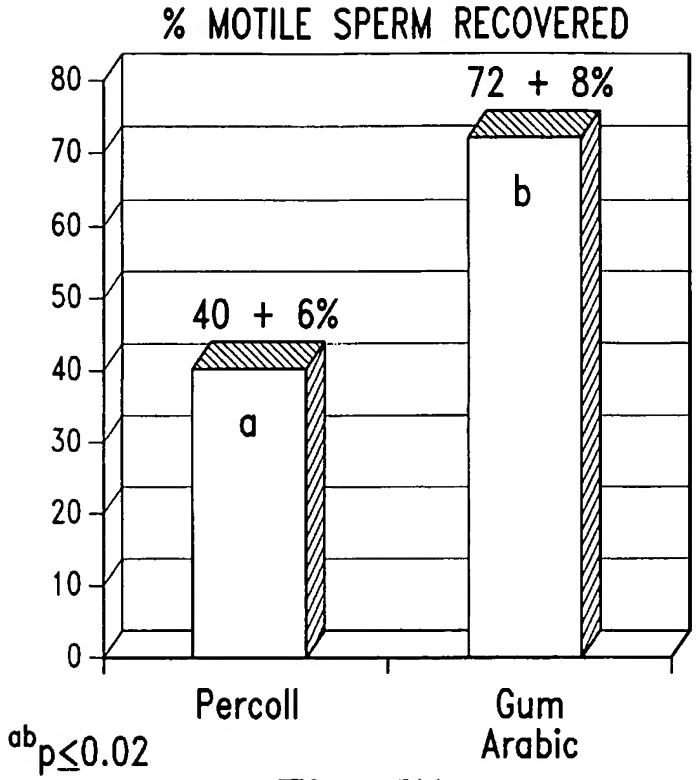

% MOTILE SPERM RECOVERED
80
70
60
50
40
30
20
10
0
72 + 8%
b
40 + 6%
a
Percoll
Gum Arabic
ab p≤0.02

Fig. 7A

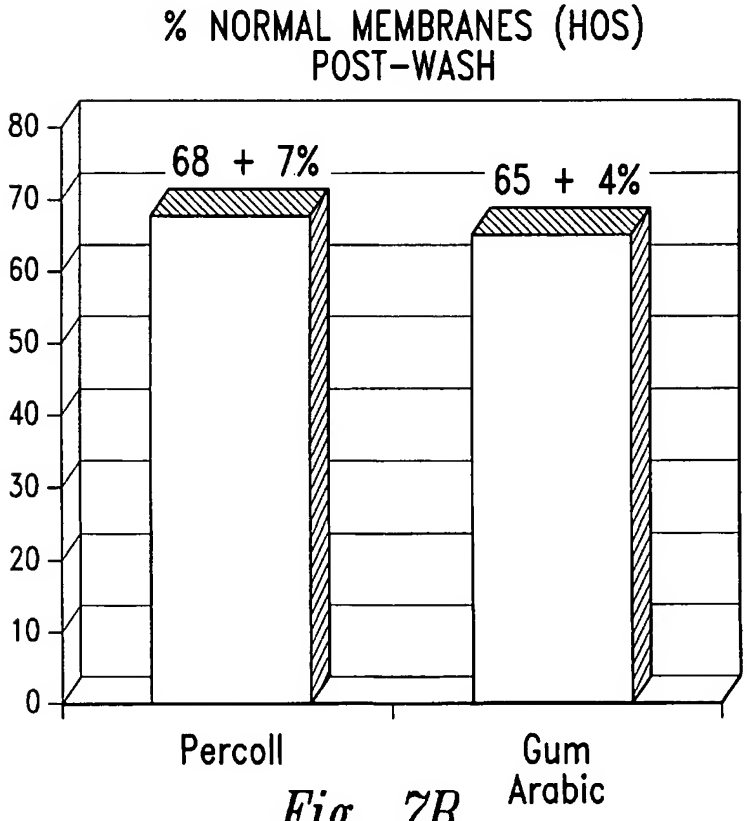

% NORMAL MEMBRANES (HOS) POST-WASH
80
70
60
50
40
30
20
10
0
68 + 7%
65 + 4%
Percoll
Gum Arabic

Fig. 7B

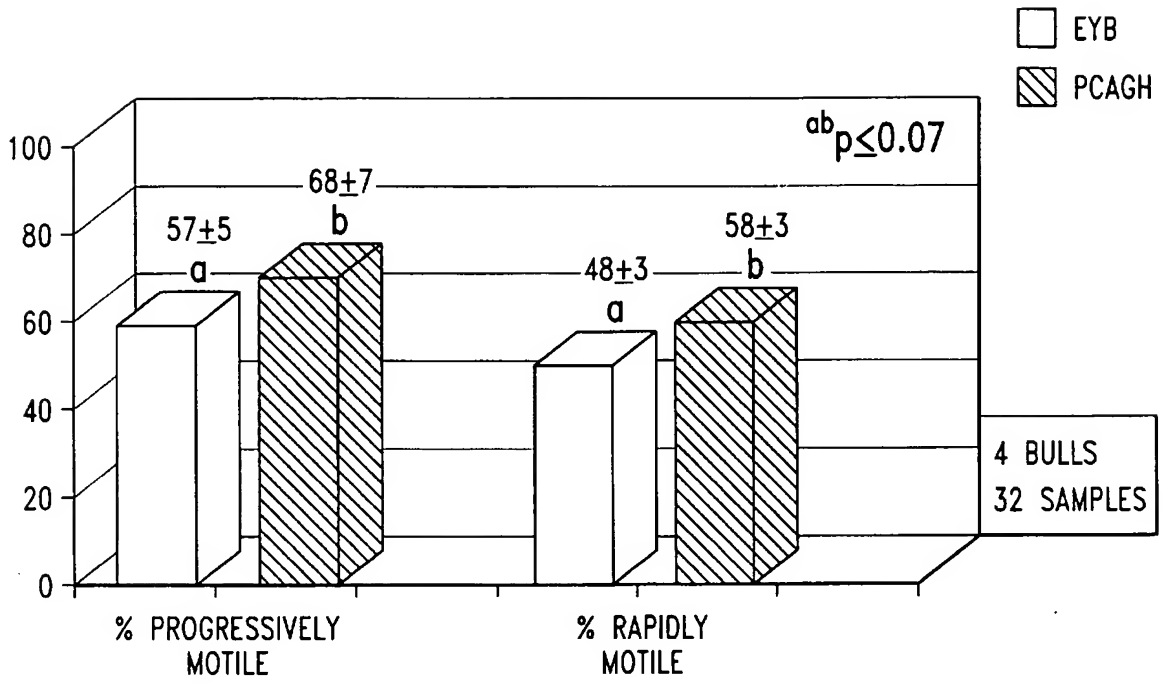

EYB
PCAGH
$^{ab}p \leq 0.07$
100
80
60
40
20
0
57±5
a
68±7
b
48±3
a
58±3
b
4 BULLS
32 SAMPLES
% PROGRESSIVELY MOTILE
% RAPIDLY MOTILE

Fig. 8

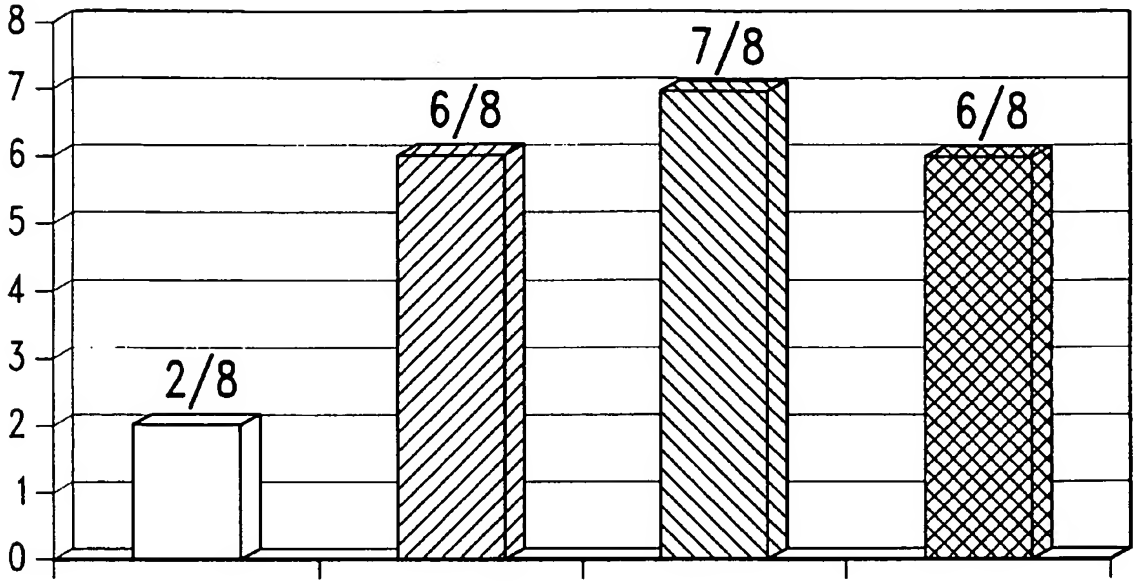

8
7
6
5
4
3
2
1
0
2/8
6/8
7/8
6/8
Standard EYB + glycerol
Na Citrate, lecithin, pectin, BSA, glycerol
EYB, pectin, taxol, glycerol
EYB, arabinogalactan, taxol, glycerol

Fig. 9

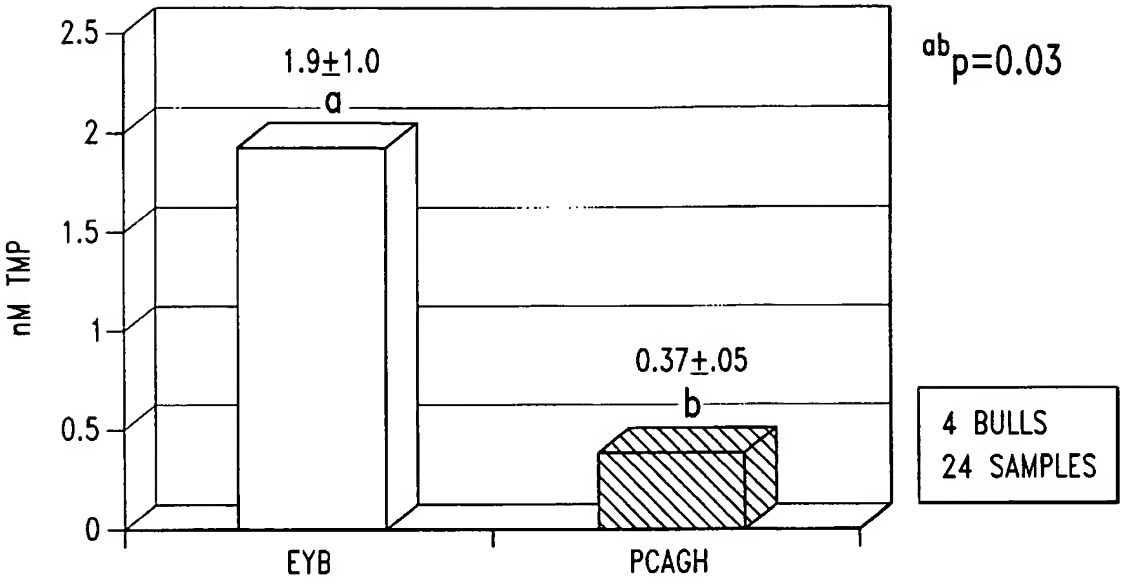

2.5
2
1.5
1
0.5
0
nM TMP
1.9±1.0
a
0.37±.05
b
EYB
PCAGH
abp=0.03
4 BULLS
24 SAMPLES

Fig. 10

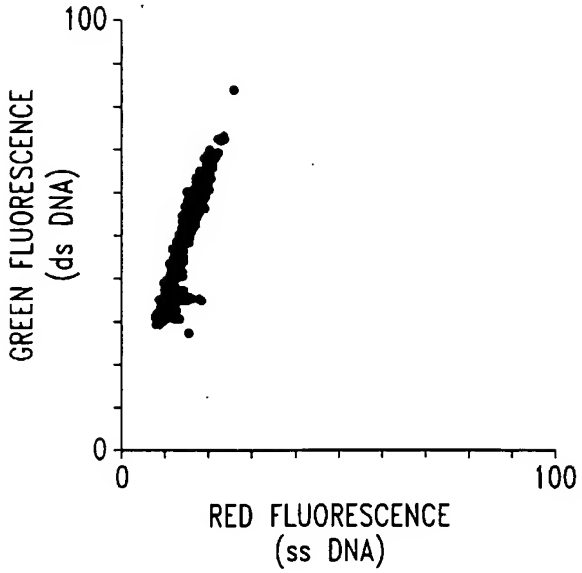


Fig. 11A

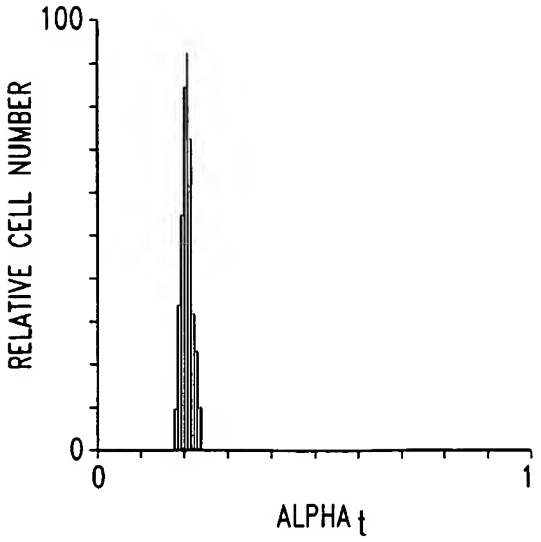


Fig. 11B

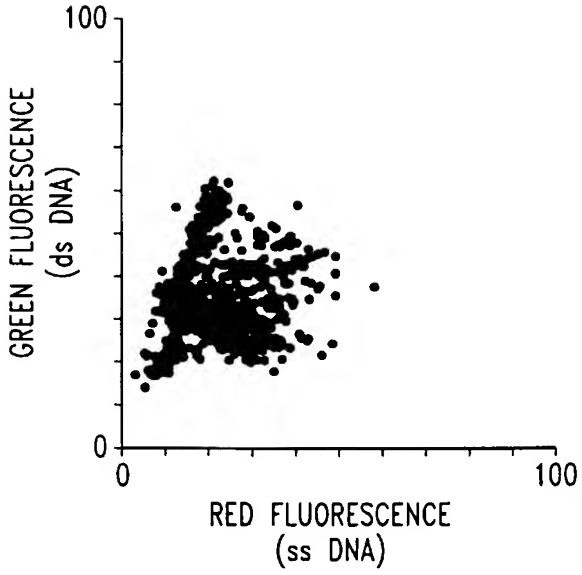


Fig. 11C

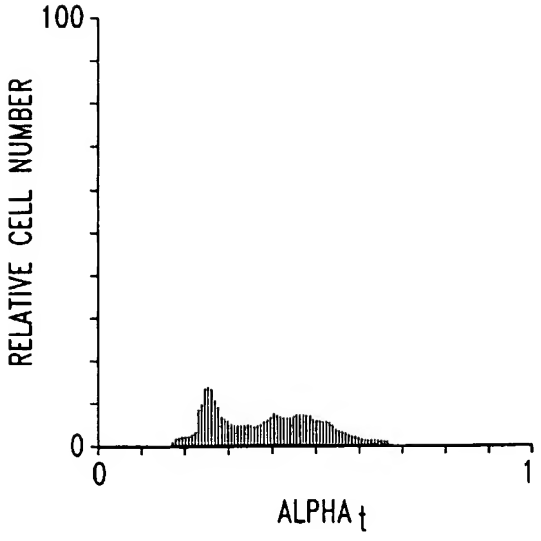


Fig. 11D

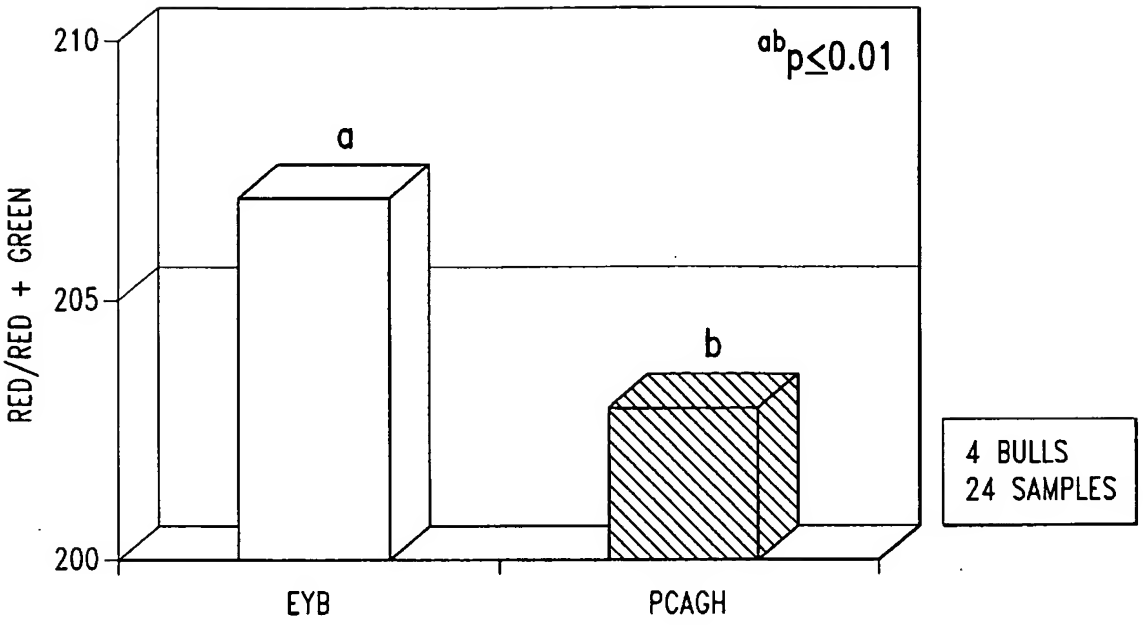

210
205
200
RED/RED + GREEN
$^{ab}p \leq 0.01$
a
b
EYB
PCAGH
4 BULLS
24 SAMPLES

Fig. 12

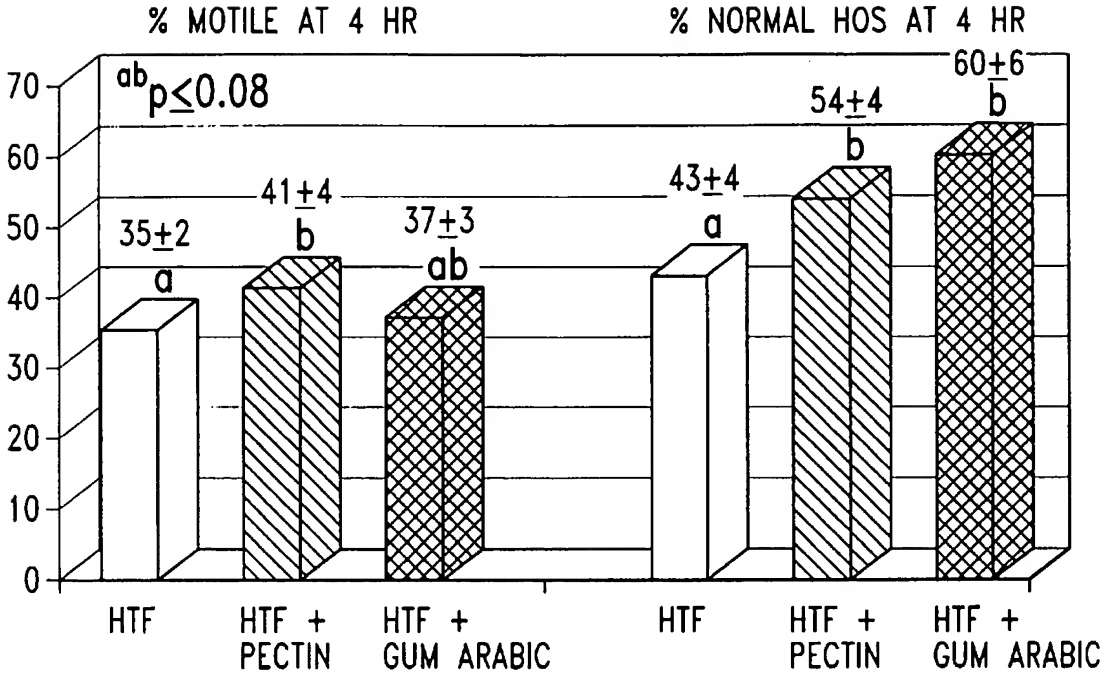

% MOTILE AT 4 HR
% NORMAL HOS AT 4 HR
ab p≤0.08
70
60
50
40
30
20
10
0
35±2
a
41±4
b
37±3
ab
43±4
a
54±4
b
60±6
b
HTF
HTF + PECTIN
HTF + GUM ARABIC
HTF
HTF + PECTIN
HTF + GUM ARABIC

Fig. 13

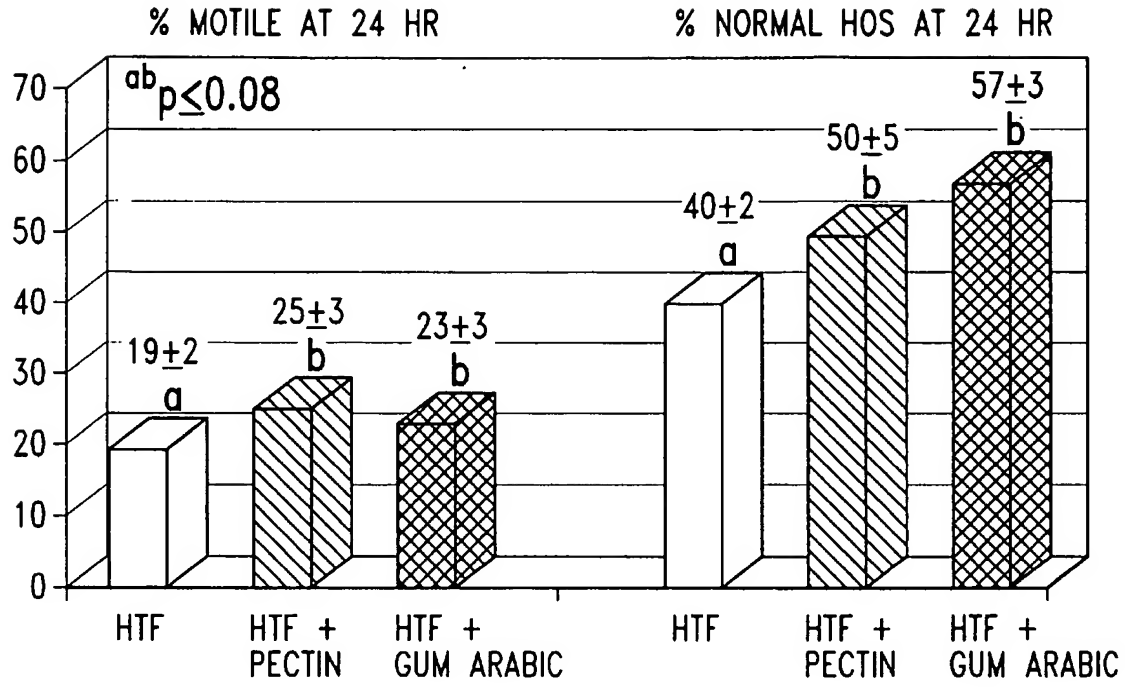

% MOTILE AT 24 HR
% NORMAL HOS AT 24 HR
ab p≤0.08
70
60
50
40
30
20
10
0
19±2
a
25±3
b
23±3
b
40±2
a
50±5
b
57±3
b
HTF
HTF + PECTIN
HTF + GUM ARABIC
HTF
HTF + PECTIN
HTF + GUM ARABIC

Fig. 14

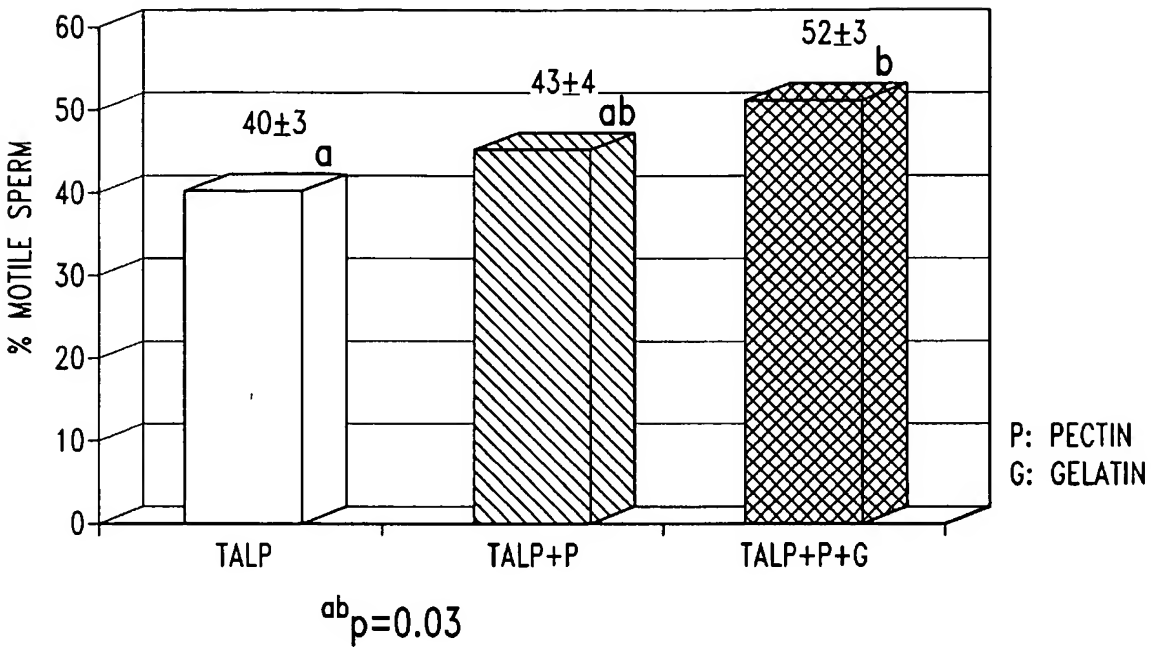

60
50
40
30
20
10
0
% MOTILE SPERM
40±3
a
43±4
ab
52±3
b
TALP
TALP+P
TALP+P+G
abp=0.03
P: PECTIN
G: GELATIN

Fig. 15

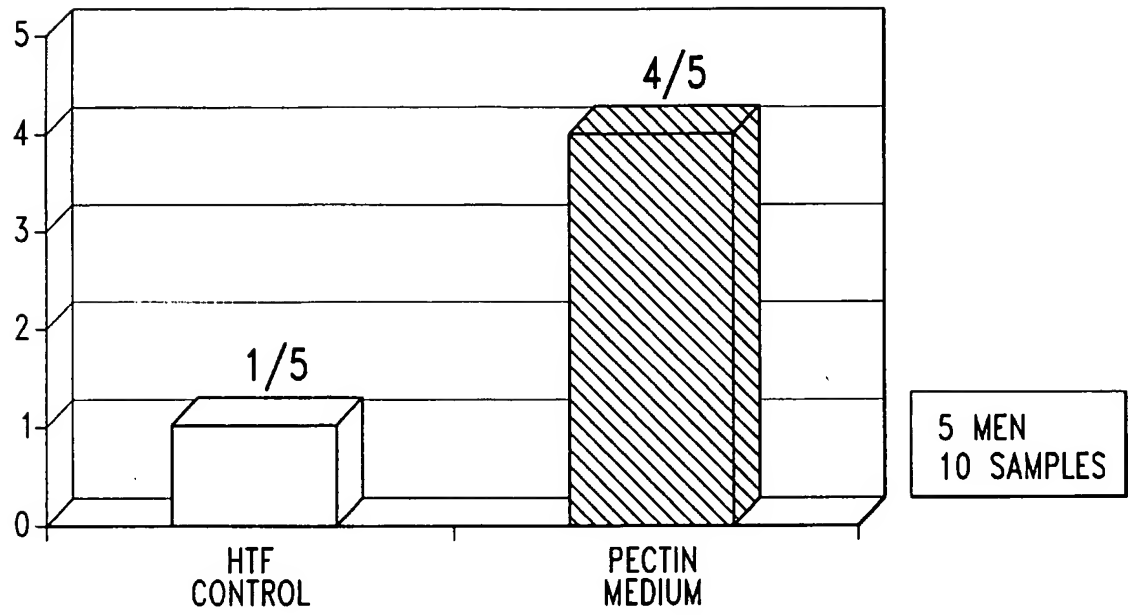


Fig. 16

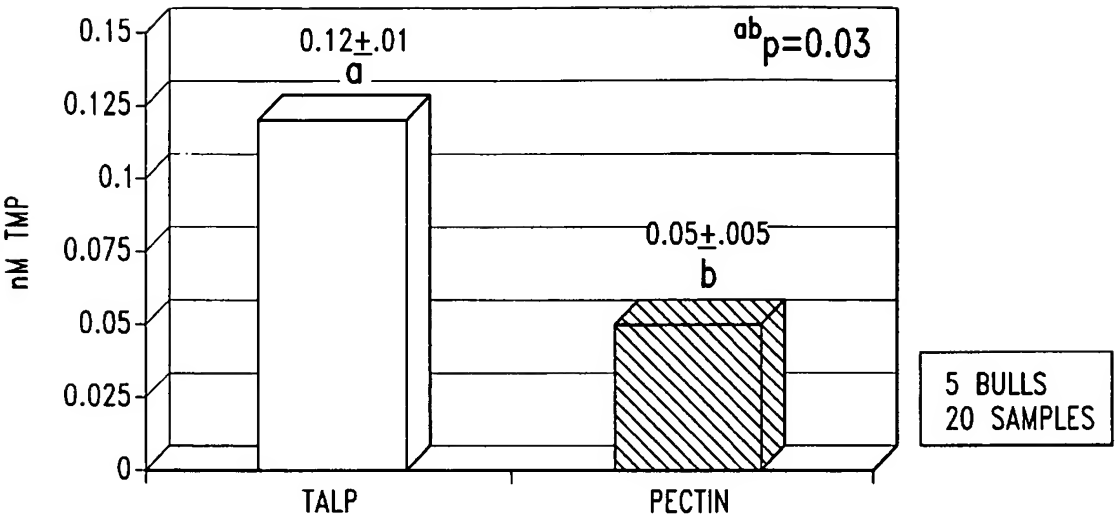

0.15
0.125
0.1
0.075
0.05
0.025
0
nM TMP
0.12±.01
a
ab p=0.03
0.05±.005
b
TALP
PECTIN
5 BULLS
20 SAMPLES

Fig. 17

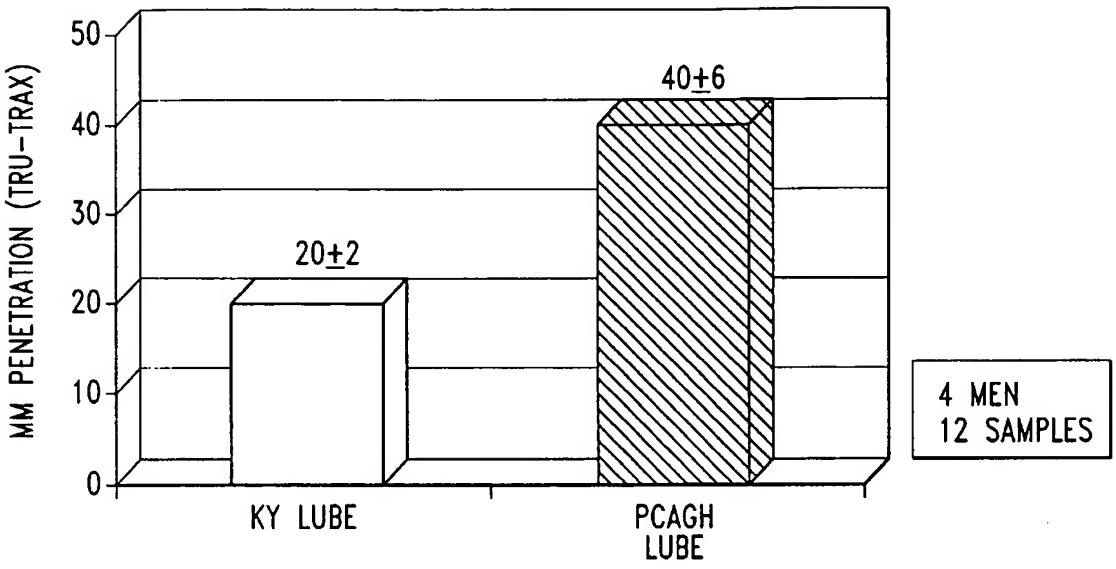

MM PENETRATION (TRU-TRAX)
50
40
30
20
10
0
20±2
40±6
KY LUBE
PCAGH LUBE
4 MEN
12 SAMPLES

Fig. 18

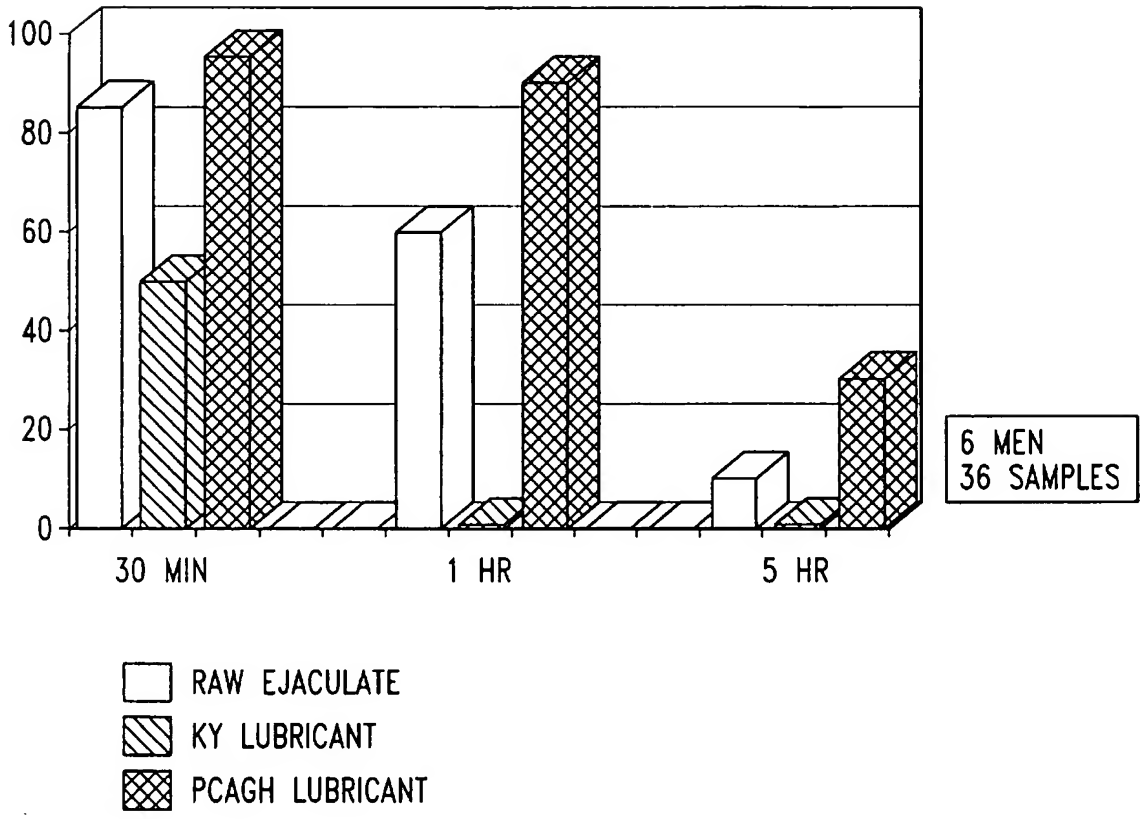

100
80
60
40
20
0
30 MIN
1 HR
5 HR
6 MEN
36 SAMPLES
RAW EJACULATE
KY LUBRICANT
PCAGH LUBRICANT

Fig. 19

METHODS AND COMPOSITIONS TO IMPROVE GERM CELL AND EMBRYO SURVIVAL AND FUNCTION

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/007,081, filed Oct. 19, 1995.

TECHNICAL FIELD

The present invention relates generally to the use of polysaccharides containing arabinose, galactose and/or hexuronic acid in promoting in vivo and in vitro survival and improved function of sperm, oocytes, and embryos.

BACKGROUND OF THE INVENTION

In nature, fertilization occurs by sperm cells being deposited into the female of warm-blooded animal species (including humans) and then binding to and fusing with an oocyte. This fertilized oocyte then divides to form an embryo. Over the last several decades, the use of assisted reproduction techniques has allowed scientists and clinicians to intervene in these events to treat poor fertility in some individuals or to store sperm, oocytes or embryos for use at other locations or times. The procedures utilized in these cases include: washing a sperm sample to separate out the sperm-rich fraction from non-sperm components of a sample such as seminal plasma or debris; further isolating the healthy, motile (swimming) sperm from dead sperm or from white blood cells in an ejaculate; freezing or refrigerating of sperm (storage) for use at a later date or for shipping to females at differing locations; extending or diluting sperm for culture in diagnostic testing or for use in therapeutic interventions such as in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI); culturing or freezing oocytes from the female for use in in vitro fertilization; and culturing or freezing of embryos prior to transfer back to a female in order to establish a pregnancy.

At each step of the way, in vitro intervention decreases the normal survival and function of sperm, oocytes, and embryos. Much research has been dedicated towards improving these procedures; however, overall success remains limited. For example, <20% of IVF attempts result in the birth of a child. Additionally, only half or less of sperm cells routinely survive the freezing process, such that pregnancy rates with frozen sperm from donors average between 10 and 20%. Oocytes and embryos also show significantly disrupted function after culture or freezing. Specifically, human oocytes survive the freezing process at very low levels. Thus, in spite of several decades of work, much room remains for improvement in the field of assisted reproduction technologies and especially in gamete and embryo handling, culture, and storage.

One common procedure used in sperm collection is washing sperm cells. Washing sperm prior to its use in assisted reproduction technologies is important for a variety of reasons. An ejaculate contains seminal plasma in addition to sperm cells, and the sugars and proteins in seminal plasma can be toxic to sperm cells after ejaculation. Also, sperm samples that have been frozen contain cryopreservation media which needs to be washed from the sperm cells prior to insemination in the female of some species, particularly birds and women. For all species, cryopreservative media cause lipid membrane peroxidation (LPO) and degeneration of the sperm after thawing. Generally, washing involves

centrifuging a sample of semen or thawed sperm through a diluting wash media, which allows collection of a sperm-rich pellet. Although a very common procedure, centrifugation itself can cause sperm lipid peroxidation and membrane breakdown.

After a sperm wash process, or in place of it, a specific procedure for the isolation of the motile sperm from a sample may be done. An ejaculate contains dead and dying sperm that release enzymes that can damage the live, motile sperm. In addition, an ejaculate contains white blood cells, red blood cells, and bacteria which are also toxic to the healthy sperm in an ejaculate. Sperm isolations involve separating out the live, healthy, and motile sperm for use in diagnostic or therapeutic procedures. Generally, sperm are isolated by allowing the motile sperm to swim away from the dead sperm and debris (sperm swim-up), by centrifuging the sperm through a density gradient, or by passing the sperm through a column that binds the dead sperm and debris. Each of these techniques has its own disadvantages. Swim-up only recovers low sperm numbers, and it requires a long culture period. Current centrifugation gradient reagents are generally toxic to sperm, such that an added wash step is necessary to remove the gradient solution from the sperm sample. Column methods have poor selectivity for motile sperm and do not always result in good recovery of sperm numbers from a full ejaculate.

Once sperm have been washed or isolated, they are then extended (or diluted) in culture or holding media for a variety of uses. Existing sperm culture techniques result in losses of motile sperm and also damage sperm DNA over time in culture. Although sperm survive for days in the females of most species, sperm survival in culture is typically only half as long as that seen in vivo, and sperm from males with poor quality ejaculates may survive for even shorter time periods in culture. Much of this damage is due to lipid peroxidation of the membrane and DNA or chromatin breakdown. Sperm are extended in media for use in sperm analysis and diagnostic tests; assisted reproduction technologies, such as IVF, gamete intrafallopian transfer, or ICSI; insemination into the female; and holding prior to cryopreservation. Each of these uses for extended or diluted sperm requires a somewhat different formulation of basal medium; however, in all cases sperm survival is suboptimal outside of the female reproductive tract.

Likewise, oocytes and embryos often develop abnormally (e.g., chromosome number, cytoskeleton formation) in culture compared to in vivo conditions. Additionally, current culture methods utilize high doses of animal proteins, like serum, which may result in an oversized fetus and perinatal complication for the offspring.

Some of the difficulties in assisted reproduction technologies can be overcome by coculturing sperm, oocytes and embryos with cell feeder layers. However, cocultures are of variable quality and variable reliability and add the risk of pathogen transfer from the feeder cells to the gametes or embryos that are to be transferred back to living animals or humans.

Storage of sperm is of widespread importance in commercial animal breeding programs, human sperm donor programs and in dealing with some disease states. For example, sperm samples may be frozen for men who have been diagnosed with cancer or other diseases that may eventually interfere with sperm production. Freezing and storage of sperm is critical in the area of preservation of endangered species. Many of these species have semen which does not freeze well under existing methods. In

standard animal husbandry, artificial insemination (AI) with frozen bull sperm is used in 85% of dairy cows. Because most commercial turkeys have become too heavy to mate naturally, AI is required on almost all turkey farms. Approximately six million turkey hens are inseminated each week in the United States. However, existing methods of storing collected turkey sperm cannot support sperm survival for even the several hours required to transport semen between farms, much less for long-term freezing. This limits the ability to store or transport genetic material to improve production. Human donor AI is also used for couples with severe male infertility; however, pregnancy rates with donor semen in people is only a quarter of that found with natural reproduction. Furthermore, surgical insemination may be required.

Current techniques for freezing sperm from all species result in membrane damage and subsequent death of about half of the sperm cells in a sample. Much of this damage occurs by reactive oxygen species causing lipid peroxidation of the sperm membrane. Despite these widespread and serious problems, the state of the art and protocols for this field have changed very little in the last 15 years. In light of the increasing use of frozen sperm in a variety of settings, a new method of freezing or storing sperm would offer a major breakthrough for human fertility specialists, animal producers, and conservation specialists.

Freezing oocytes and embryos is also important for preserving genetic material from endangered species, increasing offspring production from valuable livestock individuals, or for retaining embryos for infertile couples prior to transfer. Current methods of freezing oocytes and embryos are less than optimal with decreased development potential seen. In fact, human oocytes are rarely successfully frozen, necessitating placing multiple embryos into a woman's uterus which increases the number of dangerous and high risk multiple pregnancies. In addition, IVF embryos or genetically altered embryos from all species, such as those obtained after gene therapy, have very poor post-freezing survival rates with existing freezing media. This includes cloned embryos and embryos derived from embryonic stem cells (ESC).

For couples with fertility problems, an alternative course to the assisted reproduction techniques described above for improving the chance of conception is to have multiple, timed events of coitus during oocyte ovulation. For many of these couples, the emotional stress of infertility and the necessity of timed coitus month after month can lead to the need for artificial lubrication during intercourse. However, most commercially available lubricants are spermicidal, as is saliva, so that infertile couples are often instructed by their physician to not utilize any lubricant products during intercourse. In addition, many aspects of reproductive medicine in both humans and animals would be enhanced by the use of non-spermicidal lubrication during procedures such as manual sperm sample collection, artificial insemination, and uterine catheterization. The lubricant products on the market are not acceptable for use in these situations because of their spermicidal properties.

Following deposition in the female, sperm must penetrate the cervical mucus of the female and swim to the oviducts (Fallopian tubes) where they remain until ovulation and fertilization occurs. Sperm that are compromised may not be able to swim through this mucus and are thus not available for the fertilization process, limiting the fertility of the male. Furthermore, sperm that are slow to enter the cervical mucus are left in contact with the vaginal mucosa which is acidic and can inactivate sperm within several hours. Sperm with impaired fertilization potential include those that have been frozen, those where the male has antibodies in his semen that weaken the sperm, or sperm that have abnormal motion or shapes. Therapeutic options for treating male factor infertility, which accounts for 60% of infertility cases, are currently very limited and often end up utilizing very expensive intervention techniques, such as ICSI in which a single sperm is injected into an egg. As well, an increased incidence in genetic and/or birth defects have been reported for offspring from such sperm injection techniques. A product that improves sperm survival, motility and mucus penetration after ejaculation or insemination in the female could increase the number of sperm available in the oviduct for fertilization and thus could improve the chances of conception occurring without invasive intervention.

The present invention provides a variety of compositions that are non-toxic to sperm, oocytes or embryos, which additionally improve their function and survival during in vitro handling and which improve sperm function for use by couples trying to conceive naturally, as well as for use in a variety of assisted reproduction techniques in humans and animals. The present invention further provides other related advantages.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for improving the function of germ cells (sperm and oocytes), and embryos both in vivo and in vitro.

Within one aspect, methods for isolation of motile sperm having improved function are provided comprising contacting a sample containing sperm with a solution comprising a polysaccharide containing arabinose, galactose and/or hexuronic acid (PCAGH) to form a mixture, wherein the PCAGH is not arabinogalactan, and then removing the wash solution. This mixture is subjected to conditions sufficient to separate the motile sperm from the rest of the sample, thereby isolating the sperm with improved function. In a related aspect, methods for washing sperm to remove the nonsperm portion of a sample and to obtain sperm with improved function are provided comprising contacting a sample containing sperm with a solution comprising a polysaccharide containing arabinose, galactose and/or hexuronic acid, wherein the PCAGH is not arabinogalactan, and removing the wash solution. Within certain embodiments, the polysaccharide is pectin, arabic acid, gum arabic, gum ghatti, gum karaya, gum guar, galactopyranosylarabinose, galacturonic acid, gum locust bean, gum tragacanth, carrageenan, or derivatives thereof. Within another embodiment, the sample is semen. Within yet other embodiments, the sample is obtained from human, bovine, canine, equine, porcine, ovine, avian, rodent or exotic species. In certain embodiments, it may also include other density gradient compounds, such as dextran, iodixanol, sucrose polymers, nycodenz, or polyvinylpyrolidine coated silica (Percoll). In other embodiments, the solution comprises a balanced salt solution and a macromolecule.

Within another aspect, a sperm wash medium is provided comprising a polysaccharide containing arabinose, galactose and/or hexuronic acid (PCAGH) and a macromolecule wherein the PCAGH is not arabinogalactan. The polysaccharide is present at a concentration sufficient to improve sperm function at 1–50%. In certain embodiments, the macromolecule is gelatin, bovine serum albumin, human serum albumin, egg yolk, oviductin, polyvinyl alcohol, hyaluronic acid, gelatin, catalase, or casein. Generally, the solution further comprises a balanced salt solution.

Within a related aspect, a medium for the isolation of motile sperm from a sample is comprised of a PCAGH at 0.01–5% and a density gradient compound for centrifugation isolation, or a macromolecule for swim-up separation.

Within another related aspect, an extending medium for sperm is provided comprising a PCAGH in a solution at a concentration sufficient to improve sperm function.

In another aspect, a non-spermicidal lubricant for increasing fertilization potential in animals is provided comprising a non-spermicidal lubricious compound and a polysaccharide containing arabinose, galactose and/or hexuronic acid (PCAGH). Within certain embodiments, the lubricious compound comprises glycerine, methylcellulose, propylene glycol, plant oils, or petroleum jelly, or a combination of glycerin and petroleum jelly, or a combination of polyethylene oxide, sodium carboxypolymethylene and methylparaben. Within other embodiments, the polysaccharide is pectin, arabinogalactan, arabic acid, gum arabic, gum ghatti, gum karaya, gum guar, galactopyranosylarabinose, galacturonic acid, gum locust bean, gum tragacanth, carrageenan, or a derivative thereof. The lubricant may be used in vivo by administration or placement in a vagina prior to coitus or artificial insemination, or used during semen collection, such as by applying the lubricant to a penis prior to ejaculation into a receptacle or collecting sperm into a receptacle containing the lubricant. In a related aspect, the lubricant is used to lubricate medical devices or a hand prior to reproductive procedures.

In yet other aspects of the subject invention, methods for increasing the survival of sperm, oocyte, embryo and embryonic stem cells in vitro are provided comprising contacting a sample containing one of the cell types with a medium acceptable to the cell and including a polysaccharide containing arabinose, galactose and/or hexuronic acid (PCAGH). Within certain preferred embodiments, the medium is a balanced salt solution medium. Within other embodiments, the medium further comprises a macromolecule, such as blood serum, synthetic serum supplements, bovine serum albumin, human serum albumin, oviductin, superoxide dismutase, vitamin E, gelatin, polyvinyl alcohol, hyaluronic acid, catalase, chondroitin sulfate, heparin, egg yolk, skim milk, casein, melanin, hormone or growth factors. With other embodiments, the medium also comprises a sperm stimulant. Sperm stimulants include caffeine, follicular fluid, oxytocin, kallikrien, prostaglandins, thymus extract, pentoxyfilline, deoxyadenosine, inositol, platelet activating factor, hypotaurine, or mercaptoethanol.

Within yet other aspects, methods for reducing the loss of functional sperm, reducing the cellular damage to an oocyte, and reducing the cellular damage to an embryo or embryonic stem cell (ESC), resulting from the storage of the cells in a refrigerated, frozen or vitrified state are provided. More specifically, a polysaccharide containing arabinose, galactose and/or hexuronic acid (PCAGH) and a sample containing sperm, oocyte, or embryo, are combined wherein the polysaccharide is in an amount effective to reduce the loss or damage and the sample is then stored in a refrigerated, frozen or vitrified state. Within certain preferred embodiments, an additional cryoprotective compound is added. Within a related aspect, a medium for storing sperm, oocytes, or embryos is provided comprising a balanced salt solution and a polysaccharide containing arabinose, galactose and/or hexuronic acid.

These and other aspects of the invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions. Each of these references are incorporated herein by reference in their entirety as if each were individually noted for incorporation.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a drawing depicting an anatomic overview of how a lubricant containing a polysaccharide containing arabinose, galactose and/or hexuronic acid (PCAGH) may be used.

FIG. **2** is a graph illustrating the shift of fluorescence output following DNA damage.

FIG. **3** is a chart showing the percentage of sperm with normal membranes after culture for 24 hours in HTF alone or containing various carbohydrates, including PCAGHs.

FIG. **4** is a chart illustrating lipid membrane peroxidation levels of bull sperm cultured for 4 hours with various PCAGH.

FIG. **5** is a stained electrophoretic gel of apple pectin following various enzymatic treatments.

FIG. **6** is a chart showing the percentage of bull sperm that are motile following a 2 or 24 hour culture in TALP containing various fractions of pectin.

FIG. **7** is a pair of charts demonstrating the percentage of motile sperm (left panel) and sperm with normal membranes (right panel) after washing sperm through Percoll or a buffer containing gum arabic. Semen from four ejaculates were tested.

FIG. **8** is a chart demonstrating the motility characteristics of bull sperm frozen in egg yolk buffer (EYB) extender or PCAGH extender.

FIG. **9** is a chart showing the number of bull sperm samples having >5% motility after freezing and thawing in the shown extenders, followed by 24 hours of culture.

FIG. **10** is a chart showing the extent of lipid membrane peroxidation for frozen bull sperm after holding sperm for 10 minutes after thawing in egg yolk buffer (EYB) extender or PCAGH extender.

FIGS. **11A–11D** are flow cytometry profiles of DNA from sperm frozen with PCAGH (A) and (B) or egg yolk buffer (C) and (D) extenders.

FIG. **12** is a chart illustrating the susceptibility of sperm DNA after thawing to acid or heat denaturation for sperm frozen in egg yolk buffer (EYB) or PCAGH extender.

FIG. **13** is a graph showing the percentage of human sperm which are motile or have normal membranes after 4 hours culture in HTF media with or without PCAGH.

FIG. **14** is a chart showing the percentage of human sperm that are motile and have normal membranes after 24 hour culture in HTF media with or without a PCAGH.

FIG. **15** is a chart presenting results of motility of sperm following a 5 hour culture in TALP with various additives.

FIG. **16** is a chart showing the number of men who have >5% motile sperm after 72 hour culture in HTF medium with or without a PCAGH.

FIG. **17** shows the levels of lipid peroxidation of sperm cultured for 4 hours with or without a PCAGH.

FIG. **18** is a graph illustrating penetration of bovine cervical mucus after 30 minutes incubation of raw semen with a PCAGH or KY lubricant.

FIG. **19** is a graph illustrating the progressive motility of sperm over time when incubated in semen alone or in KY lubricant or a PCAGH lubricant.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms that will be used hereinafter.

As used herein, "polysaccharides containing arabinose, galactose and/or hexuronic acid" (hereinafter referred to "PCAGH") refers to a polymer comprising arabinose and galactose or hexuronic acid or combinations thereof (e.g., a hexuronic acid and galactose or arabinose or both). A monomeric unit of a hexuronic acid (e.g., galacturonic acid) may also be used within the context of the present invention. When the polysaccharide comprises arabinose and galactose, at least a disaccharide must be present. Generally, however, PCAGHs have a molecular mass in the range of 6 kDa to 1500 kDa. The PCAGH may contain other saccharides as well, or other molecules such as proteins, peptides, lipids, nucleic acids and the like. Examples of a PCAGH include, but are not limited to arabinogalactan, pectin, arabic acid, gum arabic, fucoidan, funoran, iridophycan, gum ghatti, gum tragacanth, quince seed gum, plantago polysaccharide, psyllium seed, flax seed gum, gum karaya, gum guar, locust bean gum, carrageenan, seaweed extracts, plant or root extracts from *Gymnema sylvestre, Helianthus annuus* L., *Angelica acutiloba, Ariemisia princps, Bupleurum Falatum* L. *Panax ginseng, Malva sylvestris* var. *mauritiana, Rubus fruticosus* and *Hibiscus sabdariffa*; polysaccharides from microorganisms, polysaccharides from plant cell cultures, or derivatives of the above. As used herein, a "hexuronic acid" is a tetrahydroxy aldehyde acid obtained generally by oxidation of hexose sugars. Such hexuronic acids include glucuronic acid, galacturonic acid, mannuronic acid, guluronic acid, iduronic acid, and the like (see, "Carbohydrates," ed. P. M. Collins, Chapman and Hall, NY, 1987; Merck Index).

As used herein, "improved function" of sperm refers to the improved potential of a sperm to fertilize an oocyte. This potential may be assessed by motility, viability, survival time, membrane stabilization, levels of lipid peroxidation damage, chromatin stability, mucus penetration, oocyte fertilization or subsequent embryonic development and the like. Likewise, "improved function" of an oocyte refers to the improved potential for fertilization of the oocyte by sperm, followed by normal development. "Improved function" of an embryo refers to improved potential for normal development and offspring production. This potential for oocytes and embryos is assessed by evaluating chromosome numbers, cell numbers, cytoskeleton formation and metabolic activity. "Improved function" means that the sperm, oocyte or embryo have enhanced performance as assessed by one of these assays when treated with a PCAGH under conditions described herein as compared to a control (i.e., no treatment with a PCAGH).

As used herein, "embryo" refers to an animal in early stages of growth following fertilization up to the blastocyst stage. An embryo is characterized by having totipotent cells, which are nondifferentiated. In contrast, somatic cells of an individual are cells of a body that are differentiated and are not totipotent.

As used herein "embryonic stem cell" (ESC) refers to established cultured cell lines originating from a single embryo. ESCs are a population of cells having identical genetic material. Each cell is totipotent and, if fused with a nonfertilized oocyte, generates genetically identical animals.

I. Polysaccharides Containing Arabinose, Galactose and/or Hexuronic Acid

As described above, a polysaccharide containing arabinose, galactose and/or hexuronic acid ("PCAGH") refers to a polymer containing at least arabinose and/or galactose units in combination with a hexuronic acid or to the hexuronic acid alone (e.g., galacturonic acid). Preferred PCAGHs contain arabinose, galactose and galacturonic acid. These polysaccharides generally occur in nature as water soluble polymers obtained from the gum or pectic fractions of plants. Such substances are also released by plant cells and microbial cells in culture (Bushel et al., *Food Hydrocolloids* 1:359–363, 1987). Chemical and enzymatic fractionation of PCAGHs provide active fractions that are also useful in the present invention (see, Examples). The PCAGHs or their derivatives may be chemically synthesized in vitro. In addition, derivatives obtained through refinements such as acid or heat (e.g., autoclaving) treatments of these PCAGHs, are also useful in the present invention, examples being production of arabic acid from gum arabic and production of small molecular weight derivatives from arabinogalactan via autoclaving. Glycoproteins may also be used if they contain an active fraction. PCAGHs are commercially available in a variety of forms, such as arabinogalactan, pectin, arabic acid and gum arabic (Sigma, St. Louis, Mo.; GlycoTech, Rockville, Md.; Seikagaku, Ijamsville, Md.; Accurate Chemical Co., Westbury, N.Y.; Boehringer Mannheim, Indianapolis, Ind.).

Pectic substances, more commonly referred to as pectins, are a complex mixture of polysaccharides characterized by a backbone of $\alpha(1\rightarrow4)$ linked galacturonic acid units that are partially methyl-esterified (O'Neill et al., *Methods in Plant Biochemistry* 2:415–441, 1990). All pectins contain some associated neutral sugars, such as L-arabinose, D-galactose, L-rhamnose, D-xylose and D-glucose. Chemical and enzymatic degradation of pectins reveal long and regular uronic regions (smooth) and rhamnose-rich regions (hairy) that have neutral sugars as side chains. Pectins are present in the primary cell walls of all seed-bearing plants and are major components of dicotyledons (e.g., citrus and legumes) and gymnosperms (e.g., Douglas fir). Commercially important sources of pectin sources include apple and citrus pulps (e.g., Sigma Chemical Co., St. Louis, Mo.), sugar beet, and alfalfa.

Gum exudates are viscous fluids that are discharged from plants and contain high levels of polysaccharides. Gums are also found in various seeds, seaweed, and microbial cultures. These gums comprise polysaccharides that are complex and highly branched with residues of hexuronic acids (typically D-glucuronic acid and/or galacturonic acid) along with neutral sugars (Aspinall, *The Carbohydrates*, ed. W. Pigman, and D. Horton, Ch. 39:515–536, 1970). Examples of gums that contain PCAGHs include gum arabic, gum tragacanth, gum ghatti, gum karaya, and larch arabinogalactan. Arabinogalactans are found in most plants and are present as side chains in many gums and pectic complexes (Clarke et al., *Phytochemistry* 18:521–540, 1979). The Type II arabino-3,6-galactans have been detected in seeds, leaves roots, fruit and gum exudates. Arabinogalactan from the mountain larch has a β-D$(1\rightarrow3)$ linked galactopyranosyl backbone with 1,6 linked side chains. Derivatives of arabinogalactan, such as amino derivatives, succinyl-arabinogalactan, glutaryl-arabinogalactan, arabinogalactan hydrazide, phosphoryl arabino-galactan and the like, may be used in the present invention (see, for example, PCT application WO 93/25239). Gum arabic, an exudate from *Acacia senegal* is regarded as representative of exudate gums that

have a core composed of branched chains of D-galactopyranose residues. Gum arabic also typically contains residues of L-arabinose, D-galactose, L-rhamnose and D-glucuronic acid. Arabic acid is an acid-ethanol precipitate derivative of gum arabic. Many of these gums can be obtained commercially (e.g., Sigma Chemical Co., St. Louis, Mo.).

Hexuronic acids are 6 carbon sugars with a COOH group. The sugar may be linear or ring-structured. Side groups may be present in addition. Hexose sugars that may be oxidized include glucose, galactose, mannose, gulose, idose, talose, altrose, and allose. Common hexuronic acids include glucuronic acid, galacturonic acid, and mannuronic acid. (See, "*Carbohydrates*" supra; Merck Index for others.)

Other PCAGHs include, for example, fucoidan, fimoran, iridophycan, quince seed gum, plantago polysaccharide from psyllium seed, flax seed gum, gum guar, locust bean gum, carrageenan, seaweed extracts; plant or root extracts from *Gymnema sylvestre, Helianthus annuus* L., *Angelica acutiloba, Ariemisia princps, Bupleurum Falatum* L. *Panax ginseng, Malva sylvestris* var. *mauritiana, Rubus fruticosus* and *Hibiscus sabdariff*, polysaccharides from microorganisms;

polysaccharides from plant cell cultures; or active derivatives of the above.

Other PCAGHs may be obtained through derivation of naturally-derived pectic substances and gums by chemical and enzymatic means (see, in general, *The Carbohydrates*, Ch. 39, ed. W. Pigman et al., Academic Press, N.Y. and London, 1970; *Methods in Plant Biochemistry* 2:415, 1990; Stephen et al., *Methods in Plant Biochemistry* 2:483, 1990; Lau et al., *Carb. Res.* 168:219, 1987). Acid hydrolysis and heat autolysis procedures yield small oligomeric derivatives with biological activity. Polygalacturonic oligomers have been synthesized (Nakahara and Ogawa, *Carbohydrate Research* 200:363–375, 1990) and chemically modified (Moloshok et al., *Archives of Biochemistry and Biophysics* 294(2):731–734, 1992). Also encompassed are gums that have been modified through the introduction of neutral groups to increase solution viscosity; the addition of methyl, ethyl, hydroethyl and similar groups; the introduction of acidic groups; the introduction of graft polymers; or modification by thermal dextinization, partial hydrolysis and mild oxidation. Modification may be performed using, for example, pectinase, endoarabinanase, α-L-arabinofuranosidase, and endopolygalacturonase. Enzymes are commercially available (e.g., Megazyme, Bozeman, Mont.; Seikagaku, Ijamsville, Md.; GlycoTech, Rockville, Md.; Sigma, St. Louis, Mo.).

PCAGHs may be identified by degradation procedures, including acid hydrolysis, enzymatic digestion, combined with detection methods (e.g., GC, mass-spectrometry, TLC, NMR, IR spectroscopy) for the monomeric sugars or uronic acids. Other commonly employed methods to identify saccharides may be interchangeably substituted (see, for example, Müller and Franz, *Planta Med* 58:60, 1992; Gonda et al., *Carb. Res.* 198:323, 1990; Wicken and Lëiting, *Anal. Biochem.* 229:148, 1995; Taylor and Buchanan-Smith, *Anal. Biochem.* 201:190, 1992; Bach and Schollmeyer, *Anal. Biochem.* 203:335, 1992; Ló et al., *Carb. Res.* 255:271, 1994; de Vries et al., *Carb. Polymers* 3:193, 1983; McCleary and Metheson, *Adv. in Carb. Chem. Biochem.* 44:147, 1986; Leitão et al., *Carb. Polymers* 26:165, 1995; Eagles et al., *Phyto. Chem.* 34:709, 1993; Selvendran and Rydan, *Methods in Plant Biochemistry* 2:549, 1990).

The various polysaccharides exhibit widely different degrees of water solubility. In general, polysaccharides with high solubility are soluble to about 60% before the viscosity makes the solution essentially unworkable. Low solubility polysaccharides are soluble to about 10% or less before the viscosity makes the solution essentially unworkable. High solubility polysaccharides include arabinogalactan and gum arabic. Low solubility polysaccharides include pectin and arabic acid.

II. Improved Sperm Function

As noted above, improved sperm function refers to the increased capability of sperm to fertilize an oocyte. This function may be assayed by a broad range of measurable cell functions. Such assayable functions include sperm motility, sperm viability, membrane integrity of sperm, in vitro fertilization, sperm chromatin stability, survival time in culture, penetration of cervical mucus, as well as sperm penetration assays and hemizona assays. Sperm have improved function after exposure to a composition or method if they perform significantly better ($p<0.05$) with a PCAGH under conditions described herein as compared to a control (i.e., assay performed without including a PCAGH). A brief description of various assays that may be used to assess sperm function are presented herein. These assays are provided as exemplary techniques; variations or alternative methods that measure the tested functions may be used.

Sperm motility is one function that may be used to assess sperm function and thus fertilization potential. Motility of sperm is expressed as the total percent of motile sperm, the total percent of progressively motile sperm (swimming forward), or the speed of sperm that are progressively motile. These measurements may be made by a variety of assays, but are conveniently assayed in one of two ways. Either a subjective visual determination is made using a phase contrast microscope when the sperm are placed in a hemocytometer or on a microscope slide, or a computer assisted semen analyzer is used. Under phase contrast microscopy, motile and total sperm counts are made and speed is assessed as fast, medium or slow. Using a computer assisted semen analyzer (Hamilton Thorn, Beverly, Mass.), the motility characteristics of individual sperm cells in a sample are objectively determined. Briefly, a sperm sample is placed onto a slide or chamber designed for the analyzer. The analyzer tracks individual sperm cells and determines motility and velocity of the sperm. Data is expressed as percent motile, and measurements are obtained for path velocity and track speed as well.

Sperm viability is measured in one of several different methods. By way of example, two of these methods are staining with membrane exclusion stains and measurement of ATP levels. Briefly, a sample of sperm is incubated with a viable dye, such as Hoechst 33258 or eosin-nigrosin stain. Cells are placed in a hemocytometer and examined microscopically. Dead sperm with disrupted membranes stain with these dyes. The number of cells that are unstained is divided by the total number of cells counted to give the percent live cells. ATP levels in a sperm sample are measured by lysing the sperm and incubating the lysate with luciferase, an enzyme obtained from fireflies, which fluoresces in the presence of ATP. The fluorescence is measured in a luminometer (Sperm Viability Test; Firezyme, Nova Scotia, Canada). The amount of fluorescence in the sample is compared to the amount of fluorescence in a standard curve allowing a determination of the number of live sperm present in the sample.

Membrane integrity of sperm is typically assayed by a hypo-osmotic swell test which measures the ability of sperm to pump water or salts if exposed to non-isotonic environ-

ments. Briefly, in the hypo-osmotic swell test, sperm are suspended in a solution of 75 mM fructose and 25 mM sodium citrate, which is a hypo-osmotic (150 mOsm) solution. Sperm with intact, healthy membranes pump salt out of the cell causing the membranes to shrink as the cell grows smaller. The sperm tail curls inside this tighter membrane. Thus, sperm with curled tail are counted as live, healthy sperm with normal membranes. When compared to the total number of sperm present, a percent of functional sperm may be established.

The degree of membrane integrity is preferably determined by lipid peroxidation (LPO) measurements which assess sperm membrane damage generated by free radicals released during handling. Lipid membrane peroxidation is assayed by incubating sperm with ferrous sulfate and ascorbic acid for one hour in a 37° C. water bath. Proteins are precipitated with ice-cold trichloroacetic acid. The supernatant is collected by centrifugation and reacted by boiling with thiobarbituric acid and NaOH. The resultant malondialdehyde (MDA) formation is quantified by measuring absorbance at 534 nm as compared to an MDA standard (M. Bell et al., *J. Andrology* 14:472–478, 1993). LPO is expressed as nM MDA/10^8 sperm. A stabilizing effect of PCAGHs results in decreased LPO production.

The stability of chromatin DNA is assayed using the sperm chromatin sensitivity assay (SCSA). This assay is based on the metachromatic staining of single and double stranded DNA by acridine orange stain, following excitation with 488 nm light. Green fluorescence indicates double strand DNA, and red fluorescence indicates single strand DNA. The extent of DNA denaturation in a sample is expressed as α and calculated by the formula α=red/(red+green). In all cases, sperm are mixed with TNE buffer (0.01 M Trisaminomethane-HCl, 0.015M NaCl, and 1 mM EDTA) and flash frozen. Sperm samples are then subjected to 0.01% Triton-X, 0.08N HCl and 0.15M NaCl, which induces partial denaturation of DNA in sperm with abnormal chromatin. Sperm are stained with 6 g/ml acridine orange and run through a flow cytometer to determine α.

In vitro fertilization rates are determined by measuring the percent fertilization of oocytes in vitro. Maturing oocytes are cultured in vitro in M199 medium plus 7.5% fetal calf serum and 50 μg/ml luteinizing hormone for 22 hours. Following culture for 4 hours, the sperm are chemically capacitated by adding 10 IU of heparin and incubated with oocytes for 24 hours. At the end of the incubation, oocytes are stained with an aceto-orcein stain or equivalent to determine the percent oocytes fertilized. Alternatively, fertilized oocytes may be left in culture for 2 days, during which division occurs and the number of cleaving embryos (i.e., 2 or more cells) are counted.

Survival time in culture of sperm (time to loss of motility) is another convenient method of establishing sperm function. This parameter correlates well with actual fertility of a given male. Briefly, an aliquot of sperm is placed in culture medium, such as Tyrode's medium, pH 7.4 and incubated at 37° C., 5% CO_2, in a humidified atmosphere. At timed intervals, for example every 8 hours, the percentage of motile sperm in the culture is determined by visual analysis using an inverted microscope or with a computer assisted sperm analyzer. As an endpoint, a sperm sample is considered no longer viable when less than 5% of the cells have progressive motility.

Another parameter of sperm function is the ability to penetrate cervical mucus. This penetration test can be done either in vitro or in vivo. Briefly, in vitro, a commercial kit containing cervical mucus (Tru-Trax, Fertility Technologies, Natick, Mass.), typically bovine cervical mucus, is prepared. Sperm are placed at one end of the track and the distance that sperm have penetrated into the mucus after a given time period is determined. Alternatively, sperm penetration of mucus may be measured in vivo in women. At various times post-coitus, a sample of cervical mucus is removed and examined microscopically for the number of sperm present in the sample. In the post-coital test, improved sperm function is established if more sperm with faster velocity are seen in the mucus sample after exposure to a PCAGH lubricant versus a sample of mucus from the patient after exposure to a control lubricant.

Other assays of sperm function potential include the sperm penetration and hemizona assays. In the sperm penetration assay, the ability of sperm to penetrate into an oocyte is measured. Briefly, commercially available zona free hamster oocytes are used (Fertility Technologies, Natick, Mass.). Hamster oocytes are suitable in this assay for sperm of any species. Capacitated sperm, such as those cultured with bovine serum albumin for 18 hours, are incubated for 3 hours with the hamster oocytes. Following incubation, oocytes are stained with acetolacmoid or equivalent stain and the number of sperm penetrating each oocyte is counted microscopically. A hemizona assay measures the ability of sperm to undergo capacitation and bind to an oocyte. Briefly, in this assay, live normal sperm are incubated in media with bovine serum albumin, which triggers capacitation. Sperm are then incubated with dead oocytes which are surrounded by the zona pellucida, an acellular coating of oocytes. Capacitated sperm bind to the zona and the number of sperm binding is counted microscopically.

III. Lubricants

As noted above, within one aspect of the present invention, PCAGHs are formulated as a nonspermicidal lubricant for improving sperm function and potential fertility in animals. The lubricants comprise a base containing a lubricious compound, which is nonspermicidal, and a PCAGH.

The base of the lubricant is a nonspermicidal lubricious compound. Such lubricants include petroleum jelly, vegetable oil, glycerin, polycarbophil, hydroxyethyl cellulose, methylcellulose, silicon oil, carbomer (e.g., carbomer 934), alginate, methylparaben, palm oil, cocoa butter, aloe vera, other plant oils, alginate propylene glycol, unibase (Warner-Chilcott), mineral oil, a combination of polyethylene oxide, sodium carboxypolymethylene and methylparaben, and the like. A base lubricant of 50% petroleum jelly/50% glycerin is preferred. Additional ingredients, such as pH stabilizers and anti-oxidants, may be added. Sodium hydroxide is preferably added to bring the pH to 7.4. Other pH stabilizers include EDTA or zwitterionic buffers (e.g., TES, PIPES, MOPS, HEPES). Anti-oxidants, or free-radical scavengers such as vitamin E, may be added. In certain embodiments, silicon oil or polyvinyl alcohol are added.

A PCAGH is added to the lubricious compound to 0.01–40% (e.g., 0.01–30%; 0.01–20%), preferably to 0.1 to 5% for high viscosity polysaccharides, and most preferably to 0.1 to 1%; preferably to 1–20% for low viscosity polysaccharides, and most preferably to 10–20%. Examples of preferred embodiments include 0.1% pectin, 1% galacturonic acid, 1% gum guar, 10% gum arabic, or 20% arabinogalactan.

The lubricant is preferably non-irritating and easily applied. It may be in the form of a gel, foam, cream, jelly, suppository (see, e.g., U.S. Pat. No. 4,384,003 to Kazmiroski), or the like. The lubricant may be packaged in a kit containing a tube of lubricant and an applicator for

intra-vaginal application. For use during coitus or artificial insemination, the lubricant may be applied intra-vaginally. It may also be applied to a penis for use during intercourse or for collection of sperm. Generally, sperm donors collect sperm samples by manual manipulation without the benefit of lubrication because available lubricants and saliva are spermicidal. The lubricant of the present invention may be applied directly to the penis, coat the interior or exterior of a condom, or be placed in a receptacle for sperm collection such as a vial, tube, baggie, or other collection device.

In addition, the lubricant may be used in various assisted reproductive techniques and diagnostic procedures. For example, the lubricant may be used to coat a catheter for insertion into a bladder to collect sperm from a retrograde ejaculation. It may be used to lubricate a catheter, pipette or hand, prior to performing embryo transfer, artificial insemination, or diagnostic procedures such as endoscopy, contrast radiography or biopsy. The lubricant may be used in any animal species for sperm collection, coitus, assisted reproductive techniques and the like. Such animals include, but are not limited to, humans, bovine, equine, canine, ovine, avian, feline, and various exotic or rare species (e.g., elephant, lion, rhinoceros).

IV. Isolating and Washing Sperm

In other aspects of this invention, methods are provided for washing and isolating sperm and sperm-containing samples to obtain sperm-rich samples and samples of the most motile sperm. Such samples contain sperm with improved function. Sperm are washed by contacting a sample containing sperm with a solution containing a PCAGH, wherein the polysaccharide is not arabinogalactan. Motile sperm are isolated by contacting a sample containing sperm, such as an ejaculate, with a media solution comprising a PCAGH, wherein the polysaccharide is not arabinogalactan, and subjecting the mixture to conditions sufficient to separate the sperm.

For all these methods, the PCAGH is preferably added to a standard balanced salt solution. Such media include, but are not limited to, Tyrode's albumin lactate phosphate (TALP), human tubal fluid (HTF; Fertility Technology, Natick, Mass.), Ham's F10, Ham's F12, Earle's buffered salts, Biggers, Whitten and Whitingham (BWW), CZB, T6, Earle's MTF, KSOM, SOF, and Benezo's B2 or B3 media. Formulas for these media are well known, and preformulated media may be obtained commercially (e.g., Gibco Co. or Fertility Technologies, Natick, Mass.). In addition, a zwitterionic buffer (e.g., MOPS, PIPES, HEPES) may be added. The PCAGH includes, but is not limited to, any of the polysaccharides discussed above. Preferably the PCAGH is pectin, gum guar, or gum arabic for isolating and washing sperm. Wash media contain PCAGH at concentrations of about 1–50% (e.g., 5–30%; 5–20%; 10–20%). In preferred embodiments, gum arabic is added to about 20% or gum guar is added to about 5%. In another embodiment, galacturonic acid is added.

These media may further contain a macromolecule as long as the solution remains a balanced salt solution. Such macromolecules include polyvinyl alcohol, albumin (bovine serum albumin or human serum albumin), oviductin (Gandolfi et al., *Repro. Fert. Dev.* 5:433, 1993), superoxide dismutase, vitamin E, gelatin, hyaluronic acid, catalase, egg yolk, casein, or other protein. Albumin or gelatin is added generally at 0.5% and hyaluronic acid or polyvinylalcohol at 1.0%; the other macromolecules are added at similar concentrations (e.g., 0.05–5%). Sperm isolation media contain at least one PCAGI at about 0.01–5% (e.g., 0.1–5%, 0.1–1%, 1%–5%) in addition to either a density gradient compound for centrifugation methods, or a macromolecule for swim-up isolation methods. Density gradient materials are generally added to a concentration of 5–90%. Such materials include dextran, iodixanol, sucrose polymers, nycodenz, or polyvinylpyrrolidone coated silica (i.e., Percoll). In typical applications, a sperm containing solution is layered over a gradient material, preferably Percoll at 30–90% mixed with 0.05% pectin, and then subjected to centrifugation to collect sperm with improved function. When sperm swim-up is used to isolate sperm, a macromolecule, such as those discussed above, is added. Preferably 1–10 mg/ml of hyaluronic acid is used. A preferred medium is PCAGH at 0.01–5% (e.g., 0.05% gum arabic or 1% galacturonic acid) in combination with hyaluronic acid. Media used in any of these procedures may further comprise a balanced salt solution.

As noted above, sperm are washed or isolated by contacting a sample with a solution comprising a PCAGH, wherein the polysaccharide is not arabinogalactan, and subjecting the mixture to conditions sufficient to separate the desired sperm from the sample. Briefly, cells are contacted with the solution by placing cells in the solution from a brief time up to incubation for 4 hours. Preferably the temperature at which contacting occurs is from about 20° C. to about 39° C. Following this initial contact, different methods may be used to isolate sperm, such as centrifugation, swim-up, separation columns, and the like. For example, one such method is centrifugation of a sperm sample through a continuous gradient of the solution comprising a PCAGH. In this method, the solution comprising a PCAGH is placed in a centrifuge tube and a semen sample or sperm cells are layered over the medium at approximately a ratio of one part semen (or sample) to one part medium. The tube is centrifuged at approximately 300×g for ten to twenty minutes. A sperm-rich fraction with improved function, and thereby increased fertilization potential, is recovered in a pellet at the bottom of the tube. Because the PCAGH is non-toxic to sperm, a follow-up wash step to remove the PCAGH is not required. Isolation may be performed in a method similar to the above wash process; however, the PCAGH solution can either be layered under the sperm sample, but on top of a density gradient like Percoll, or mixed directly into the Percoll gradient. Alternatively, sperm are isolated by a swim-up method. Briefly, sperm swim-up tubes are prepared by placing 1.5 ml of wash media in a 12×75 mm round bottom tube. Sperm are layered under this wash media using a 27 gauge needle and 1 ml syringe at 1 part sperm suspension to 2 parts wash medium. The tubes are incubated undisturbed for 1 hr. After incubation, the wash medium (that the motile sperm have swum up into) is removed and centrifuged for 10 min at 300×g. A final pellet of motile sperm is then recovered for analysis or use. Other methods, such as column separation, may alternatively be used.

Sperm may be further washed after isolation of sperm by the methods described herein or by other methods used, such as centrifugation through a Percoll gradient. Washing sperm can be used to transfer sperm from one solution to another comprising a PCAGH.

For any of these methods, the sample may be semen, partially purified sperm, or purified sperm. Moreover, sperm suitable in the present invention may be procured from animal species including human, bovine, canine, equine, porcine, ovine, rodent, avian or exotic animals, such as lions, tigers, giraffes, monkeys, zebras, pandas, jaguars, elephants, rhinoceros, and others.

V. Extending and Culturing Sperm Cells and Culturing Oocytes or Embryos

In other aspects of this invention, methods for extending sperm (e.g., to dilute or suspend the sperm) to obtain sperm with improved function are provided. Sperm are extended by addition of a solution comprising a PCAGH. The concentration of PCAGH for extending sperm is from 0.001–5% (0.01–5%; 0.05–1%; 0.05–0.5%). For example, pectin, gum ghatti, or gum arabic are added at 0.05%; gum guar, galacturonic acid, or galactopyranosylarabinose at 0.1%; and arabic acid or arabinogalactan at 0.5%. Galacturonic acid may also be used alone or with other PCAGH at 0.01–5.0% (e.g., 0.01%–1%; 0.05–0.5%; 0.1–1%).

Extending sperm is used to resuspend a sperm pellet following isolation or washing, to dilute a semen sample, to dilute a culture of sperm, and the like. In this way, sperm are placed into a medium suitable for a variety of procedures, including culture, insemination, assays of fertilization potential as described herein, in vitro fertilization, freezing, intrauterine insemination, cervical cap insemination, and the like. The sperm may be added to the medium or the medium may be added to the sperm. Preferably, the medium contains gum guar, gum arabic, pectin or galacturonic acid, although another PCAGH may be used. In other aspects of this invention, methods are provided for the culture of such extended sperm to increase their survival during holding or culture at a range of temperatures from about room temperature (e.g., 20° C.) to about body temperature (e.g., 37° C. or 39° C.). This includes culture of sperm in toxicity screen tests and the holding of sperm for sorting into X and Y chromosome-containing fractions by flow cytometry for generating sexed offspring. In other aspects of this invention, sperm extending medium is used for preparing sperm for direct insemination, cryopreservation, and for intracytoplasmic sperm injection (ICSI) which requires a more viscous media to slow motile sperm down for pick-up by the transfer pipette for injection into the egg. In ICSI, the medium contains PCAGH at higher levels than a routine extender medium (ie., 1% arabic acid or 5% gum arabic) to increase viscosity. A viscous solution of PCAGH also has a positive effect on sperm function by limiting membrane damage and possible chromatin breakdown during in vitro handling. Additional embodiments include encapsulation of the sperm (Munkittrick et al., *J. Diary Sci.* 75:725–731) in an alginate or protamine sulfate microcapsule containing PCAGH, such as pectin at 0.05%. Encapsulation allows for shedding of sperm over an increased time frame so that insemination does not have to be as well timed with ovulation. PCAGH stabilizes sperm membranes from breakdown observed with current procedures.

For all these methods, except encapsulation, the polysaccharide containing arabinose, galactose and/or hexuronic acids is preferably added to a balanced salt solution which may contain zwitterionic buffers, such as TES, HEPES, PIPES, or other buffers, such as sodium bicarbonate. Sample media include, but are not limited to, TALP or HTF. Additional ingredients may include macromolecules such as those discussed herein, for example, albumin, oviductin, gelatin, hylauronic acid, milk, egg yolk, hormones, free radical scavengers (e.g., melanin, vitamin E derivatives, thioredoxine), enzymes (e.g., SOD, catalase), growth factors (e.g., EGF, IGF, PAF, VIP), polymeric molecules (e.g., heparin, dextran, polylysine, PVP or PVA). Additionally, such media may include sperm motility stimulants such as caffeine, follicular fluid, calcium, oxytocin, kallikrien, prostaglandins, thymus extracts, pentoxyfilline, 2-deoxyadenosine, inositol, flavanoids, platelet activating factor, hypotaurine, chondroitin sulfate, and mercaptoethanol. Preferred stimulants are caffeine (e.g., 5 mM) and pentoxyfilline (e.g., 1 mM). Antibiotics and antimycotics may also be included.

In other aspects of this invention, methods are provided for increasing the survival of oocytes, embryos or embryonic stem cells (ESC) in in vitro culture systems. Oocytes, embryos, or ESC are cultured for use in various diagnostic and toxicology assays, in vitro fertilization, or for the propagation of offspring. These methods comprise contacting a sample containing an oocyte, an embryo or ESC with a culture medium that includes a PCAGH.

In general, the medium for extending sperm or culturing sperm, oocytes, embryos or ESC is a balanced salt solution, such as M199, Synthetic Oviduct Fluid, PBS, BO, Test-yolk, Tyrode's, HBSS, Ham's F10, HTF, Menezo's B2, Menezo's B3, Ham's F12, DMEM, TALP, Earle's Buffered Salts, CZB, KSOM, BWW Medium, and emCare Media (PETS, Canton, Tex.). In one embodiment, M199 medium is preferred for culturing oocytes. In certain embodiments, TALP or HTF is preferred for sperm culture medium, and CZB is preferred for embryo culture medium.

The concentration of the PCAGH in the oocyte or embryo medium ranges from 0.001–5% (0.01–5%; 0.05–1%; 0.05–0.5%; 0.1–5%; 0.1–1%). Optionally, other additives may be present such as amino acids (e.g., glutamic acid) or free radical scavengers. Generally, the additives are a macromolecule, a buffer, an antibiotic and possibly a sperm stimulant if fertilization is to be achieved. As well, a hormone or other protein may be added. Such hormones and proteins include luteinizing hormone, estrogen, progesterone, follicle stimulating hormone, human chorionic gonadotropin, growth factors, follicular fluid and oviductin, albumin and amino acids. Generally, the medium also contains serum from about 1% to 20%. Preferably, serum is from the same animal source as the oocyte or embryo. Sperm, oocytes, or embryos are cultured in the media described above in 5% CO_2 and humidified air at 37° C. Cultures may contain a feeder layer comprising somatic cells, generally irradiated cells, cultured cells, or cells with a limited life span in culture (e.g, thymocytes).

VI. Freezing Sperm, Oocytes or Embryos

As noted above, in other aspects of this invention, methods are provided for reducing losses of functional sperm, reducing cellular damage to an oocyte, or reducing cellular damage to an embryo or ESC (embryo stem cell) resulting from storage in a refrigerated, frozen or vitrified state. The methods comprise combining a PCAGH in an amount effective to reduce loss or damage with a sample containing sperm, oocyte, embryo or ESC, and storing the sample in a refrigerated, frozen or vitrified state.

Sperm, oocytes, embryos, and ESC may be obtained in a variety of ways, such as described herein (see Examples). Cryoprotective medium is typically added slowly to the cells in a drop wise fashion. Such medium is prepared by adding an effective concentration of a PCAGH to a simple medium such as Tris buffer or sodium citrate buffer for sperm, PBS for oocytes or embryos, and a balanced culture medium such as M199 for ESC. The PCAGH is generally added at 0.005–30% (e.g., 0.05–20%, 0.05–10%, 0.05–5%, 0.1–10%, 0.1–5%, 1–5%), or for example, at 0.05% for pectin, 0.1% galacturonic acid, 1.0% arabic acid, 5% gum arabic or <5% for arabinogalactan. Alginate is not included.

In addition, a cryoprotective compound is optionally included. Such cryoprotective compounds include permeating and nonpermeating compounds. Most commonly, DMSO, glycerol, propylene glycol, ethylene glycol, or the

like are used. Other permeating agents include propanediol, dimethylformamide and acetamide. Nonpermeating agents include polyvinyl alcohol, polyvinyl pyrrolidine, anti-freeze fish or plant proteins, carboxymethylcellulose, serum albumin, hydroxyethyl starch, Ficoll, dextran, gelatin, albumin, egg yolk, milk products, lipid vesicles, or lecithin. Adjunct compounds that may be added include sugar alcohols, simple sugars (e.g., sucrose, raffinose, trehalose, galactose, and lactose), glycosaminoglycans (e.g., heparin, chrondroitin sulfate), butylated hydroxy toluene, detergents, free-radical scavengers, and anti-oxidants (e.g., vitamin E, taurine), amino acids (e.g., glycine, glutamic acid), and flavanoids and taxol (preferably 0.5–5 μm). Glycerol is preferred for sperm freezing, and ethylene glycol or DMSO for oocytes, embryos or ESC. Typically, glycerol is added at 3–15%; other suitable concentrations may be readily determined using the methods and assays described herein. Other agents are added typically at a concentration range of approximately 0.1–5%. Proteins, such as human serum albumin, bovine serum albumin, fetal bovine serum, egg yolk, skim milk, gelatin, casein or oviductin, may also be added.

Following suspension of the cells in the cryoprotective medium (e.g., for storage), the container is sealed and subsequently either refrigerated or frozen. Briefly, for refrigeration, the sample is placed in a refrigerator in a container filled with water for one hour or until the temperature reaches 4° C. Samples are then placed in Styrofoam containers with cool packs and may be shipped for insemination, in the case of sperm, the next day. If the sample is to be frozen, the cold sample is aliquoted into cryovials or straws and placed in the vapor phase of liquid nitrogen for one to two hours, and then plunged into the liquid phase of liquid nitrogen for long-term storage or frozen in a programmable computerized freezer. Frozen samples are thawed by warming in a 37° C. water bath and are directly inseminated or washed prior to insemination. Other cooling and freezing protocols may be used. Vitrification involves dehydration of the oocyte or embryos using sugars, Ficoll, or the like. The oocyte or embryo is then added to a cryoprotectant and rapidly moved into liquid nitrogen.

Within the present invention, sperm, oocytes, or embryos may be prepared and stored as described above. Refrigeration is generally an appropriate means for short-term storage, while freezing or vitrification are generally appropriate means for long or short-term storage.

VII. Administration and Uses

The compositions and methods of the present invention increase fertility of animals. These methods are generally applicable to many species, including human, bovine, canine, equine, porcine, ovine, avian, rodent and others. Although useful whenever fertilization is desired, the present invention has particular use in animals and humans that have a fertilization dysfunction in order to increase the likelihood of conception. Such dysfunctions include low sperm count, reduced motility of sperm, and abnormal morphology of sperm. In addition to these dysfunctions, the methods and compositions of the present invention are useful in artificial insemination procedures. Often, in commercial breedings, the male and female are geographically distant requiring the shipment of sperm for insemination. Because of the extended period of time between ejaculation and insemination, shipment in refrigerated or frozen state is necessary. As well, for particularly valuable or rare animals, long-term storage may be desirable. For humans, geographical distance or time considerations may necessitate storage of sperm. Men with diseases where radiation treatment is part of therapy or prior to vasectomies may desire to have sperm stored for future use. After frozen storage, cells are often cultured during end use. Survival and health of the cells in culture have been shown to be improved by addition of a PCAGH to the cryopreservative medium.

The lubricant is useful for all situations involving sperm collection, coitus, and artificial insemination. Currently, sperm collection is done without any lubrication because of the spermicidal nature of commercial lubricants and saliva (Goldenberg et al., *Fertility and Sterility* 26:872–723, 1975, Scoeman & Tyler, *J. Reprod. Fert.* 2:275–281, 1985,Miller et al., *Fert. and Steril.* 61:1171–1173, 1994). The use of a non-spermicidal lubricant containing a compound that improves sperm function and increases potential fertility is desirable for the comfort of the donor. As such, the lubricant may be applied to condoms or other collection devices, such as catheters or vials. Infertile couples often have the need for lubricants due to the stress of timed coitus and difficulty in conception. However, because lubricants are spermicidal, they are not recommended for use. In these cases, the application of a lubricant intravaginally, with or without an applicator, would be desirable and beneficial because sperm function would be increased (FIG. 1). Similarly, the lubricant may be applied intravaginally prior to artificial insemination to improve the chances of conception. In either case, normal sperm should swim into the cervical mucus within three minutes of coitus, with a maximum number found in the cervix three hours post-coitus. The acidic environment of the vagina inactivates sperm left in the vagina over a four-hour time period. Intravaginal application of the lubricant product improves sperm survival in the vagina and increases cervical mucus penetration.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

Example 1

Media for Sperm Isolations and Culture

Sperm function assays such as sperm motility, viability and functional membrane health (HOS) are used to determine and/or compare the biological activity of a PCAGH. Sperm samples from a male donor are obtained either from a fresh ejaculate of raw semen or a refrigerated or frozen sample processed by washing or extending as described herein. Basal medium is used throughout as follows: glucose-free TALP (Table 1) is prepared for bovine sperm, TALP supplemented with glucose (5 mM glucose) is prepared for other animal species, and human tubal fluid (HTF) from a powder mix or from a recipe (Table 2) is prepared for separation of human sperm. To the base medium, PCAGH is added, such as 0.05% pectin, 0.1% galacturonic acid, 0.5% arabic acid or 0.05% gum arabic. The medium is then filtered through a 0.2μ filter.

TABLE 1

GLUCOSE FREE TALP

Ingredient	g/500 ml
NaCl	2.922
KCl	0.1156
$NaHCO_3$	1.0500
$NaH_2PO_4H_2O$	0.0200

TABLE 1-continued

GLUCOSE FREE TALP

Ingredient	g/500 ml
Na Lactate (60% syrup)	1841 μl
$CaCl_2 \cdot 2H_2O$	0.1546
$MgCl_2 \cdot 6H_2O$	0.0407
Phenol Red	0.0050
HEPES	1.1915
BSA Fraction V	3.0
Gentimycin Sulfate	500 μl
Na Pyruvate	25 ml

Adjust pH to 7.2, filter (0.2μ; pH will adjust up to 7.4) and store at 5° C.

TABLE 2

MODIFIED HUMAN TUBAL FLUID

Ingredient	mM
NaCl	97.6
KCl	4.7
$MgSO_4 \cdot 7H_2O$	0.2
Na lactate	21.4
Na pyruvate	0.33
$NaHCO_3$	25.0
$CaCl_2 \cdot 2H_2O$	2.04
Glutamine	1.0
EDTA	0.1

Adjust pH to 7.2, filter (0.2μ; pH will adjust up to 7.4) and store at 5° C.

Example 2

Sperm Functional Assays

Sperm Count in a Suspension. Sperm cells are suspended in culture medium or a freezing medium. Numbers in a suspension are counted either manually using a hemocytometer or Makler, or by an automated Coulter counter system, a spectrophotometer, or a computer assisted semen analyzer (CASA). For example, 6 μl of sperm suspension are placed on a Makler chamber (Fertility Technologies, Natick, Mass.). The number of sperm counted in 10 squares is equivalent to the number of sperm/ml in the original suspension. Appropriate dilutions are made so that at least 100 sperm are counted.

Sperm Morphology. Sperm morphology or shape is determined by smearing a 10 μl aliquot of sperm sample at approximately 25×10^6 cells/ml onto a slide and staining with a differential stain such as Wright Giemsa at 0.1% (w/v), for 30 minutes. Sperm are then observed under a microscope and categorized as to normal or abnormal shapes (morphology); (Kruger et al., *Urology* 30:248, 1987); or by CASA sorting into normal or abnormal shapes based on computerized image analysis (Davis, *Infertility & Reproductive Medicine Clinics* 3:341, 1992).

Sperm Motility. Sperm motility measurements may be performed by subjective visual determination using a phase contrast microscope to group sperm into total percent motile (swimming), and total percent progressively motile (swimming forward). Also the speed of those sperm which are progressively motile is determined, i.e., fast, medium, slow.

Alternatively, CASA can be used to objectively determine the motility characteristics of individual sperm cells in a sample (Davis, *Infertility & Reproductive Medicine Clinics* 3:341, 1992). A 7 μl sperm sample is placed onto a slide or chamber designed for CASA, and the computer tracks individual sperm cells and determines their motility as to speed over distance. Data is then expressed as percent motile, and specific measurements are given for parameters, such as mean path velocity and track speed. The measurements of velocity and linearity correlate with future fertility in several species studied.

Sperm Viability. Sperm viability, or the percent of live sperm in a sample, is determined by membrane exclusion stains, such as Hoechst stain 33258 or eosin-nigrosin. Dead sperm stain positive because the membranes are disrupted, allowing the stain to penetrate the cells. For example, 10 μl of eosin nigrosin stain (American College of Theriogenologists, Hastings, Nebr.) is mixed with 10 μl of sperm sample. This mixture is then smeared across the slide and the number of pink (dead) and white (live) sperm are determined. Viability is expressed as the number of live cells divided by the total number of live and dead cells.

ATP levels in a sperm sample may also be utilized to determine viability. These are measured using a simple luminometer and a firefly enzyme which fluoresces when in contact with active ATP from living sperm cells (Sperm Viability Test by Firezyme, Nova Scotia, Canada). Comparing the amount of fluorescence to a standard curve allows one to determine the number of live sperm present in sample.

Membrane Function of Sperm. Functional membrane health of a sperm cell, determined by the hypo-osmotic swell test (HOS), involves putting sperm into a solution with too few salts (hypo-osmotic). This triggers sperm with healthy membranes to pump salt out of the cell and causes the membranes of the sperm to shrink as the cell grows smaller. The sperm tail then curls inside this tighter membrane. Sperm with a curled tail are the sperm which are healthy and have functional membranes. A hypo-osmotic solution of 75 mmol/L fructose and 25 mmol/L sodium citrate is prepared. One ml of this solution is added to 100 μl of sperm sample. After incubation for 30 minutes, a 10 μl aliquot of the mixture is placed on a slide and the percentage of sperm with curled tails is determined out of 100 sperm evaluated (Jeyendran et al., *J. Reprod. Fert.* 70:219, 1984).

Lipid Membrane Peroxidation of Sperm. Damage to the sperm membrane by reactive oxygen species can also be determined by measuring lipid membrane peroxidation. Sperm are incubated in 0.63% ferrous sulfate and 0.23% ascorbic acid for one hour in a 37° C. water bath. Proteins are precipitated with ice-cold 40% trichloroacetic acid. The supernatant is collected by centrifugation at 3500×g for 25 min. in the cold and reacted by boiling for 10 min. with 2% thiobarbituric acid in 0.05N NaOH. The resultant malondialdehyde (MDA) formation is quantified by measuring absorbance at 534 nm as compared to an MDA standard. Lipid peroxidation is expressed as nM MDA/10^8 sperm. Frozen, thawed sperm have increased rates of LPO as compared to freshly ejaculated sperm. (Bell et al., *J. Andrology* 14:472–478, 1993). However, freezing sperm in a PCAGH-containing medium decreases the lipid peroxidation as compared to existing methods.

Zona Binding Assay. The ability of sperm to undergo capacitation (a biochemical change in sperm which must occur prior to fertilization), and bind to an oocyte can be measured using a zona binding assay (Franken et al., *Fert. Ster.* 59:1075, 1993). In this test live, normal sperm are incubated under conditions which trigger capacitation. Bull sperm are incubated with 10 IU/ml of heparin in TALP for 4 hours. Sperm are then incubated for 1 hour with dead

oocytes, which are surrounded by the acellular coating called the zona pellucida. Capacitated sperm bind to the zona and the number binding are counted under the microscope. This number correlates with the number of normal capacitated sperm in a sample and with fertility of a sperm sample.

Sperm Penetration Assay. This test is conducted to determine the ability of sperm to penetrate into the oocyte (Rogers et al., *Fert. Ster.* 32:664, 1979). Zona-free hamster oocytes are used to perform this test for sperm of any species. Capacitated sperm (1×10^5 sperm in 100 μl of BWW medium) are incubated with the hamster oocytes for 3 hours. The oocytes are then stained (with 1% aceto-lacmoid) and the number of sperm penetrating each one is counted.

Sperm Survival in Culture. Survival in culture is determined by placing an aliquot of 1×10^6 sperm in 2 cm^2 wells with 500 μl of TALP or HTF medium in an incubator at 37° C. in 5% CO_2 and air. At timed intervals (e.g., every 8 hours), the percentage of motile sperm in the well are determined visually using an inverted microscope. Also, the forward speed is determined (fast, medium, slow). A sample is determined to be no longer viable when less than 5% of the sperm have progressive motility.

Sperm Chromatin Sensitivity Assay. This assay is based on the metachromatic staining of single and double stranded DNA by acridine orange stain, following excitation with 488 nm light, green fluorescence comes from double strand DNA and red from single strand (FIG. 2). The extent of DNA denaturation in a sample is seen as alpha=red/red+green as evaluated by the mean of alpha, the SD of alpha and the coefficient of variation for alpha. In all cases sperm to be studied are mixed with a TNE buffer (0.01 mol/L Trisaminomethane-HCl, 0.15 NaCl, and 1 mM EDTA) and flash frozen. Sperm samples are then subjected to 0.1% Triton-X, 0.08N HCl, 0.15 NaCl. This induces partial denaturation of DNA in sperm with abnormal chromatin. Sperm are then stained with 6 μg/ml acridine orange and run through a flow cytometer to determine the α value.

Sperm Function in the Female. The ability of sperm to survive and function in the female can be determined by the percent of oocytes that are fertilized in a superovulated female (unusually large numbers of oocytes ovulated due to hormonal stimulation with follicle stimulating hormone). Oocytes are retrieved from the oviduct soon after fertilization by the sperm (at approximately 24 hours). Fertilization is assessed by staining with 1% aceto-orcein. Alternatively, embryos resulting from fertilized oocytes are retrieved from the uterus several days after fertilization and counted. The ability of sperm to survive and function in the female is also determined by the numbers of accessory sperm bound to an oocyte recovered from the oviduct or uterus. The number of sperm able to reach an oocyte and bind to it, even if they are not involved in fertilization itself, is highly correlated to sperm fertility from a sample (Dejarnette et al., *J. Am. Sci.* 70:484, 1992).

In Vitro Fertilization. In vitro fertilization rates are determined by maturing oocytes in vitro in M199 media with 50 μg luteinizing hormone/ml (Brackett and Zuelke, *Theriogenology* 39:43, 1993). Following incubation, sperm are capacitated with heparin (bull sperm) or by an 18 hour incubation with albumin containing medium (human sperm) and incubated with oocytes for 24 hr. Oocytes are then stained with a 1% aceto-orcein stain to determine the percent fertilized, or left in culture to divide and the number of forming embryos are counted.

Cervical Mucus Penetration of Sperm. The ability of sperm to penetrate reproductive tract mucus of the female is measured in vitro by exposing sperm to a track containing cervical mucus (Tru-Trax, Fertility Technologies, Natick, Mass.) and measuring the distance the sperm have penetrated through the mucus at time specific intervals. An in vivo post coital test involves recovery of cervical mucus from the female with a speculum at 3–6 hrs post coitally. The number of sperm with good motility per high power field should be >10 if sperm function and cervical mucus are normal.

Example 3

Methods to Determine Sperm Function

Samples containing sperm are incubated at 37° C. (human) or 39° C. (animal) in 5% CO_2 and humidified air. At various timed intervals, sperm survival rate, motility characteristics, functional membrane health and membrane lipid peroxidation levels are determined as described in Example 2. Sperm cultured with a variety of PCAGHs (galacturonic acid, gum guar, galactopyranosylarabinose, gum karaya and gum locust bean) show superior sperm motility throughout a 24-hr culture period compared to sperm cultured in the monomeric sugar units of arabinose and galactose or in control medium with no polysaccharides (Table 3). In this same example, sperm show superior functional membrane health as determined by HOS testing (FIG. 3) and reduced levels of membrane lipid peroxidation (FIG. 4). Furthermore, sperm cultured in pectin, gum ghatti, gum arabic, arabic acid, and arabinogalactan show superior sperm motility characteristics of percent motility and forward speed over a 24-hr culture period, at the concentrations chosen, compared to sperm cultured in the PCAGH carageenan and fucoidan or in control medium without polysaccharides (Table 4).

TABLE 3

HUMAN SPERM CULTURED IN A VARTETY OF PCAGH OR THE MONOMERIC UNITS OF ARABINOSE, GALACTOSE OR GALACTURONIC ACID

Culture	Treatments								
Time	1	2	3	4	5	6	7	8	9
3 hr	=	=	=	+	+	+	±	±	c
15 hr	=	=	=	+	+	+	+	+	c
24 hr	=	=	=	+	+	+	+	+	c

(+): Superior sperm motility compared to control HTF
(=): Equivalent sperm motility compared to control HTF
(–): Inferior sperm motility compared to control HTF
Treatments:
1. arabinose
2. galactose
3. arabinose + galactose
4. galacturonic acid
5. gum guar
6. galactopyranosylarabinose
7. gum karaya
8. gum locust bean
9. control HTF

TABLE 4

NUMBER OF TIMES EACH TREATMENT SCORED AS ONE OF THE TOP THREE TREATMENTS BASED ON SPERM MOTILITY CHARACTERISTICS

	Pectin@	gum ghatti	cara-geenan^	gum arabic	fucoidan^	arabino galactan	arabic acid	control TALP
3 hr*	9+	6	0	6	0	5	6	2
6 hr*	10	5	2	7	0	4	4	1
10 hr**	6	2	1	5	0	4	3	2
24 hr*	9	3	2	6	0	4	4	2

@polysaccharides added at 0.05%
*11 replicates
**7 replicates
+Number of times each treatment scored as one of the top three treatments over eleven replicates based on sperm motility characteristics of % motile and forward speed over 24 hours of culture
§showing benefit of PCAGH over medium control.
^PCAGHs that are highly sulfated.

Example 4

Enzymatic and Chemical Fractionation of Pectin and Gum Arabic

Fractionation of Pectin by Enzymatic Digestion. 80 μg of pectin (Sigma Chemical Co., St. Louis, Mo.) are digested with endo-arabinanase from *Aspergillus niger*, α-L-arabinofaranosidase from *A. niger*, and endo-polygalacturonanase from *A. niger* (Megazyme, Bozeman, Mont.). Samples are incubated overnight at 45° C., boiled to inactivate enzyme and fractionated using Centricon 30 microconcentrators (Amicon) into a >30,000 MW and a <30,000 MW fraction. The endo-galacturonanase cleaves the polygalacturonic acid backbone yielding a variety of different MW polymers which include side chains. α-L-arabinofuranosidase cleaves arabinofuranosyl units from the reducing end of the side chains while endo-arabinanase removes the side chains from the polygalacturonic backbone. Gel fractionation of the digests shows the different size/polymer length of the oligomers following enzymatic digestion (FIG. 5).

Enzymatic fractions are dried using a Speed Vac concentrator, washed in distilled water and redried. Fractions are resuspended in HTF to 0.05% (for pectin). Cultured sperm are incubated at 37° C. in 5% CO_2 and humidified air. At various timed intervals, sperm motility characteristics are determined as described in Example 2. Enzymatic fractions greater than 30,000 MW of the endo-polygalacturonanase and endo-arabinanase, as well as the >30,000 MW of undigested pectin, stimulate superior sperm motility (both percentage of motile sperm and velocity) compared to control HTF (Table 5). Culture of sperm in fractions representing all digested fragments <30K resulted in equivocal or inferior sperm motility. Enzymatic derivatives of pectin therefore elicit different biological responses with respect to improving sperm motility during culture.

TABLE 5

SPERM MOTILITY IN PECTIN FRACTIONS

Culture	Treatments								
Time	1	2	3	4	5	6	7	8	9
3 hrs	+	=	=	=	+	+	–	–	c
17 hrs	+	±	=	=	+	+	–	–	c
24 hrs	+	=	=	=	+	+	–	–	c

(+): Superior sperm motility compared to control HTF
(=): Equivalent sperm motility compared to control HTF
(–): Inferior sperm motility compared to control HTF
Treatments
1: endo-polygalacturonanase, >30K
2: endo-arabinanase, >30K
3: undigested pectin, <30K
4: endo-polygalacturonanase, <30K
5: α-L-arabinofuranosidase, >30K
6: undigested pectin, >30K
7: α-L-arabinofuranosidase, <30K
8: endo-arabinanase, <30K
9: Control HTF

Chemical Fractionation of Pectin and Gum Arabic. Powdered commercial pectin and gum arabic (2 g; Sigma Chemical Co., St. Louis, Mo.) are suspended in 100 ml 96% ethanol and heated to 70° C. for 30 min. Alcohol soluble and insoluble fractions are then separated by centrifugation (3100×g for 15 min). The procedure is repeated three times. Alcohol insoluble fractions are air dried at room temperature overnight and extracted with dilute hydrochloric acid (0.1 M HCl, 80° C., 5 hr). Acid insoluble and soluble fractions are separated by centrifugation. The acid insoluble fraction is washed in distilled water, centrifuged and dried. A sample is taken and suspended in sperm culture to an approximate concentration of 0.05% for sperm analysis. The acid soluble fraction is dialyzed against distilled water overnight and a fraction (20 μl or 200 μl) diluted in 10 ml sperm culture medium for sperm analysis.

Sperm cultured in the above fractions are incubated at 37° C. in 5% CO_2 and humidified air. At various timed intervals, sperm survival rate, motility characteristics and functional membrane health are determined as described herein. Sperm cultured in the acid soluble fraction of acid hydrolyzed pectin and gum arabic show equivalent or superior function (as measured by sperm survival over 24 hours, sperm motility characteristics and HOS) compared to the undigested pectin and gum arabic (Table 6). The acid soluble fractions would contain small MW oligomers of the PCAGHs as well as monomeric galacturonic acid units.

TABLE 6

SPERM FUNCTION IN FRACTIONS OF PECTIN AND GUM ARABIC (N = 4 HUMAN EJACULATES)

Fractions Evaluated	Scores*
Acid Soluble Fractions:	
Pectin (20 μl/10 ml HTF)	5/9
Pectin (200 μl/10 ml HTF)	7/9
Gum Arabic (20 μl/10 ml HTF)	7/9
Gum Arabic (200 μl/10 ml HTF)	8/9
Undigested Pectin (0.05%)	8/9
Undigested Gum Arabic (0.05%)	7/9

*Data expressed as # of times out of a possible total of nine that a fraction scored better than control HTF medium. Other fractions not shown had overall scores <5/9 (total fractions evaluated = 15).

Molecular Weight Fractionation of Undigested Pectin. Powdered pectin is diluted in sperm culture medium to 0.05% and fractionated into the following MW categories by filtration centrifugation: >0.2μ, >100 kDa and <0.2μ, and <100 kD. Each fraction obtained is resuspended to the final original volume in culture medium to approximate the percentage of the fraction in the original sample.

Sperm cultured in the above fractions are incubated at 37° C. in 5% CO_2 and humidified air. At various timed intervals, sperm survival rate and motility are determined as described in Example 2. Bull sperm cultured with pectin fractions less than 100 kDa demonstrate superior motility characteristics compared to sperm cultured in media which contains pectin fractions greater than this MW (FIG. 6). Sperm motility characteristics of sperm cultured with pectin fractions >100 kDa but less than 0.2μ demonstrate inferior motility characteristics, particularly at 24 hr.

Example 5

Method of Sperm Washing

Sperm samples from a male donor are obtained either from a fresh ejaculate in raw semen or a refrigerated or frozen sample processed by washing or extending as described herein. Basal medium is used throughout as follows: glucose-free TALP (Table 1) is prepared for separation of bovine sperm, TALP supplemented with glucose (5 mM glucose) is prepared for separation of other animal sperm, and human tubal fluid (HTF) from a powder mix or from a recipe (Table 2) is prepared for separation of human sperm. Gum arabic is added to a final concentration of 20% and gelatin is added to a final concentration of 1.0%, alternately human serum albumin at 5 mg/ml can be used as the protein macromolecule.

For each species, sperm are washed by aliquoting into a centrifuge tube a volume of medium that is 1–2 times the volume of an ejaculate (ie., 3–6 ml medium for a 3 ml human ejaculate). The sample is then centrifuged at 300×g for 15 min or its centrifugal equivalent. The supernatant is aspirated off. The pellet of sperm is then resuspended with the medium of choice (depending on desired use), such as a freezing or insemination extender, or a culture medium for performing sperm functional assays as in Example 2.

The gum arabic gradient results in recovery of more of the motile sperm from the ejaculate. (FIG. 7) These sperm have superior membrane function, and subsequently live longer in culture than do sperm recovered from a Percoll gradient (Table 7).

TABLE 7

MEAN SURVIVAL IN CULTURE OF BULL SPERM AFTER SEPARATION

Percoll Control	24 ± 4 hours
Gradient	32 ± 6 hours

A continuous gradient of sperm wash product is prepared using a balanced salt medium. Glucose-free TALP is used for a bovine sperm, a glucose containing TALP for other animal species, and HTF for human sperm. A macromolecule such as human serum albumin or gelatin is added to the medium at approximately 0.5%, as is a PCAGH such as gum arabic at 20%. The concentration of macromolecule(s) and PCAGHs can be altered to accommodate the density of sperm from each species. The mixture is filtered through a 0.45 micron filter into a centrifuge tube. A semen sample is placed over the wash product at a ratio of 1 part semen to 2 parts wash product. The sample is then washed through the PCAGH product by centrifugation at 300×g for 15 min. The pellet of sperm is assayed in terms of sperm numbers recovered, morphology of recovered sperm, sperm motility, membrane function, survival time in culture and IVF rates.

An additional advantage of using PCAGH is that a follow-up wash step to remove them is not required since they are nontoxic to sperm, as is not the case of Percoll which requires a wash step. Additionally, a slight antimicrobial activity for the PCAGH is seen which could add further benefit to the washing of semen samples (Table 8).

TABLE 8

POTENTIAL ANTIMICROBIAL ACTIVITY OF PCAGH

	Staphylococcus	Streptococcus	Haemophilus
Arabinogalactan	xx	xx	xx
Pectin		xx	

XX: Zone of inhibition on a Mueller Hinton plate as demonstrated for this specific PCAGH-organism combination.

Example 6

Sperm Freezing or Refrigeration Technique

Sperm samples are obtained as fresh ejaculates. Sperm are either washed through a PCAGH containing gradient (as above) or are left in raw semen. Freezing medium is prepared using a Tris-buffered solution containing TES, Tris, sodium citrate, fructose, penicillin, streptomycin (Prins and Weidel, *Fert. Ster.* 46:147, 1986). To this solution, 20% egg yolk, 7% glycerol, and an effective amount of a PCAGH, such as 0.1% gum guar, 0.05% pectin, 1% arabinogalactan or 0.1% galacturonic acid is added. Additionally, 1 μM taxol or 0.25% methylcellulose may be added to the freeze mixture. Egg yolk free recipes may also be used (Table 9). The medium is then filtered through a 0.45μ filter. The freezing medium is added drop by drop to the raw semen until a one to one dilution has been reached. The extended sperm sample is then placed in a refrigerator until the mixture reaches 4° C. The sperm mix is aliquoted into freezing straws or cryovials, placed in liquid nitrogen vapor phase for 1 hour, and then plunged into liquid nitrogen. If chilled, sperm samples are shipped in Styrofoam containers at this time with Kool packs and mailed overnight for insemination the next day. If frozen in lN_2, sperm samples are placed in the vapor phase of lN_2 and can be mailed for next day delivery or stored.

Sperm refrigerated or frozen with PCAGH extender is assayed for function after storage by thawing the sperm sample in a 37° C. water bath and evaluating motility, viability, zona binding, membrane function, lipid peroxidation, sperm chromatin, IVF, and sperm function in the female.

Human sperm frozen with a PCAGH, as compared to that frozen with standard Tris-egg yolk (TEY), show improved function. Bull sperm recovered after freezing and thawing in the PCAGH containing extender also have superior percent motility as compared to sperm frozen in a Tris-Egg yolk extender (FIG. 8), their survival over time in culture is better (FIG. 9), and they have less lipid membrane peroxidation and chromatin damage (FIGS. 10–12).

This extender also allows sperm to be frozen without utilizing milk products or egg products which may carry pathogens and which require special handling conditions prior to sperm freezing.

TABLE 9

NON-EGG YOLK CONTAINING SEMEN EXTENDER

Ingredient	Percent
Sodium Citrate	2.9
Type IV Soy Lecithin	1
Bovine Serum Albumin	2
Pectin	0.05
Glycerol	10

Example 7

Sperm Dilution (Extension) and Culture Techniques

Sperm samples are obtained as described above (see Example 1). Basal medium is used throughout as follows: glucose-free TALP (Table 1) is prepared for separation of bovine sperm, TALP supplemented with glucose (5 mM glucose) is prepared for separation of other animal sperm, and human tubal fluid (HTF) is prepared for separation of human sperm. All supplies are purchased from Sigma, St. Louis, Mo., or Fertility Technologies, Natick, Mass. Sperm are separated from semen using a wash solution containing PCAGH or placed directly into media alone. Culture or extender medium is made by adding 5 mg/ml albumin, 0.5% gelatin or 0.1% PVA and an effective amount of PCAGH to basal medium. Specifically, PCAGH concentrations of 0.05% for pectin or gum arabic, 0.5% for arabic acid or arabinogalactan and 0.1% for gum guar or galacturonic acid are used.

Cultured sperm are incubated at 37° C.–39° C. in 5% CO_2 and humidified air. At 8-hour intervals the sperm survival rate is determined. Additionally, motility, viability and sperm penetration rates may be evaluated. Sperm cultured with 0.05% pectin or 0.05% gum arabic live longer and swim faster throughout the culture time period than sperm in control media with no PCAGH (FIGS. 13–16). They also have less lipid peroxidation and overall better membrane function (FIG. 17).

Sperm for direct transfer into a female are diluted (extended) by adding a medium to a sperm sample and transferring the diluted sperm sample into the female via a catheter. In vitro testing of the efficacy of sperm extended in this manner to penetrate cervical mucus has shown that sperm in a PCAGH medium penetrate mucus faster than do those in control medium (Table 10; FIG. 18).

TABLE 10

MEAN BOVINE CERVICAL MUCUS PENETRATION IN MM AT 30 MINUTES OF INCUBATION IN EXTENDING MEDIUM

control TALP	20 mm
arabinogalactan-containing medium	35 mm
pectin-containing medium	40 mm
arabic acid-containing medium	27 mm
gum arabic-containing medium	22 mm

Example 8

Lubricant Containing PCAGH

A base lubricant of 50% glycerine and 50% petroleum jelly is prepared. Alternately, a commercial non-toxic lubricant base such as Slippery Stuff (Wallace-Ofarrel, Puyallup, Wash.) or a mixture of polyethylene oxide, carboxypolymethylene and methylparaben is used. PCAGH is added at 0.5–1.0% for gum arabic or pectin or 5–10% for arabinogalactan. Sodium hydroxide is added to correct the pH to 7.4. In some embodiments, 0.5% polyvinyl alcohol or gelatin are added to improve sperm mucus penetration. For in vitro testing, semen samples are mixed with the PCAGH containing lube at 2 parts semen to 1 part lube. Sperm motility and viability are observed at 30 min intervals, and the mucus penetration test is done to evaluate sperm performance as compared to that seen for sperm in commercially available lubricants or in raw semen alone.

Sperm show significantly better motility over time in the glycerin and petroleum jelly lubricant containing arabinogalactan, or pectin than either KY jelly® or Priority Care (FIG. 19). KY lube has been reported to be spermicidal, but Priority Care is marketed as a "nonspermicidal" lubricant.

Sperm showed an increased ability to penetrate cervical mucus in lubricant containing 20% arabinogalactan or 1% pectin (Table 11) as well as increased penetration in a PCAGH lube compared to KY lube (FIG. 18).

TABLE 11

MEAN BOVINE CERVICAL MUCUS PENETRATION IN MM AT 30 MINUTES OF INCUBATION FOR LUBE PRODUCT

Priority Care	8 mm
Arabinogalactan Containing Lube	22 mm
Pectin Containing Lube	17 mm

Example 9

Testing of PCAGH on Vaginal Mucosa

A product containing sperm and PCAGH, such as either sperm freezing extender or lubricant, is tested for irritation of vaginal mucosa both in vitro and in vivo.

In vitro testing is conducted by incubating vaginal epithelial cell monolayers with solutions of product and evaluating (1) histological changes and (2) cell growth of vaginal epithelial cells (VEC). (1) Briefly, VEC are collected from macaque monkeys and cultured in DME: Ham's F12 (50:50) media containing 10% fetal bovine serum, growth factors (e.g., epidermal growth factor at 10 ng/ml) and antibiotics (1% antibiotic/antimycotic premix, Gibco). VEC are cultured in standard medium for 24 hours on Matrigel-coated (Collaborative Biochemical, Bedford, Vt.) coverslips placed

in wells of 24-well tissue culture plates in order to optimize polarization, differentiation and secretory capacity. Cells are then cultured with low and high concentration (e.g., 0.005% to 30%, depending on the viscosity of the PCAGH) of solutions of product for 12, 24 and 48 hours. At the end of each incubation period, coverslips are rinsed with PBS and preserved in tissue fixative. Cells are stained with hematoxylin/eosin and observed histologically for signs of cellular degeneration. (2) VEC are plated at an intermediate density (5×10^3 cells/well, 96-well tissue culture microplate) in standard culture medium. Following a 24-hour attachment period, cells are cultured in treatment (e.g., 0.005% to 30% product concentration) or control media for five days. Cell growth is determined at 24-hour intervals over the five-day treatment period using a modification of the MTT endpoint assay. In this assay system, growth is correlated to uptake of MTT by cell mitochondria and conversion to an insoluble blue formazan crystal which can be evaluated spectrophotometrically at 560 nm following solubilization in propanol (R. Mosmann, *J. Immunol. Methods* 65:55–63, 1983).

Example 10

Isolation of Oocytes, Embryos, and ESC

Sperm cells from a male donor are obtained either from a fresh ejaculate in raw semen or a refrigerated or frozen sample processed by washing or extending as described herein.

Oocytes from a female are obtained by aspiration of follicles during surgery, ultrasonic guided transvaginal aspiration, or aspiration of ovaries removed from the female. Oocytes may be obtained from fetal females, nonhormonally stimulated females (yielding immature primary oocytes), or hormonally stimulated females treated with follicle stimulating hormone or its equivalent (yielding mature, secondary oocytes).

Embryos may be obtained by in vitro fertilization (IVF) of oocytes and subsequent culture, flushing of the oviduct after fertilization and retrieval of embryos, flushing of the uterus after fertilization and retrieval of embryos, thawing of previously frozen embryos, or nuclear transfer and cloning of embryos. Cloned embryos are produced by fusing unfertilized oocytes with disaggregated cells of an existing embryo in order to produce multiple embryos, which are genetically identical.

Cloned embryos can also be obtained through the use of embryonic stem cells. Embryonic stem cells are ongoing cell lines of totipotent cells which came from an individual embryo. These cells are grown in a petri dish containing thousands of single cells, which, if fused with an inactive oocyte, can lead to the production of genetically similar animals.

Example 11

Oocyte Quality Assay

Oocyte quality is determined by the ability of the cumulus cells surrounding the oocyte to expand during incubation in M199 medium with or without 50 μg/ml luteinizing hormone for 22 hours. Normal oocytes will have >3–5 layers of expanded cumulus. Normal cumulus cell expansion is required for oocytes to perform normally in IVF.

Alternatively, oocyte quality is determined by staining the oocytes with a 1% aceto-orcein stain and determining the percentage of oocytes entering metaphase II. This is a required maturational step which allows the oocyte to have only half of the chromosome number of the female.

Example 12

Embryonic Quality Assays

Embryonic development may be evaluated by a variety of tests including normal cleavage or division of the embryo in culture (Lindner and Wright, *Theriogenology* 20:407, 1983); normal formation of a blastocyst cavity at an appropriate time in culture; counting the number and health of cells found in the embryo using Hoechst 33342 stain (Pursel et al., *Theriogenology* 24:687); transfer to a female and establishment of a pregnancy; and transfer to a female and subsequent birth of a normal offspring.

Example 13

Oocyte and Embryo Freezing Techniques

Oocytes and embryos are added to a PCAGH containing medium consisting of phosphate buffered saline, and 0.05% pectin or gum arabic. Additionally, 18% Ficoll may be added. A final concentration of 40% ethylene glycol is obtained and oocytes are rapidly vitrified by placing them in liquid nitrogen vapor prior to plunging into liquid nitrogen. Alternately, oocytes or embryos in PCAGH media are added to 3.5 M DMSO or 1.5M propanediol and then packaged in freezing straws and placed in a programmable freezer or exposed to liquid nitrogen vapor for 1–2 hours. Frozen straws are then plunged into liquid nitrogen for storage. Oocytes or embryos are evaluated for normal development in culture, and after transfer as described in Examples 11 and 12.

Freezing in PCAGH containing media allows oocytes and embryos to be frozen without the use of serum which can carry pathogens and cause concern for international shipments or transfer into women. Embryos develop with better and more normal cell numbers in PCACH freeze medium and have better pregnancy rates after transfer.

Example 14

Oocyte and Embryo Culture Techniques

Oocytes and embryos are cultured in a balanced salt medium such as CZB or M199 containing 0.005–0.1% PCAGH and amino acids. Somatic cells and/or 50 μg/ml luteinizing hormone are optionally added. Oocyte quality is determined at about 24 hours. Embryo quality is evaluated at 24 hour intervals over a one week time period.

PCAGH medium allows more oocytes to reach metaphase II in culture, and more embryos to develop with higher cell numbers during culture. Also embryos transferred after culture result in a higher pregnancy rate than that seen with embryos cultured in standard media. The ability to replace serum in this culture media diminishes the oversized development seen in offspring resulting from IVF, thought to be due to growth factors in the serum.

It will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without departing from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

What is claimed is:

1. A method for isolation of sperm with improved function, comprising:

(a) contacting a sample containing sperm with a solution comprising a hexuronic acid monomer or with a solu-

tion comprising a polymer wherein said polymer comprises hexuronic acid and arabinose or galactose or both arabinose and galactose, to form a mixture; and

(b) subjecting the mixture in step (a) to a condition sufficient to separate sperm from the sample, thereby isolating sperm with improved function.

2. The method of claim **1** wherein the solution of step (a) comprises said polymer.

3. A method for washing sperm to obtain sperm with improved function, comprising contacting a sample containing sperm with a solution comprising a hexuronic acid monomer or with a solution comprising a polymer wherein said polymer comprises hexuronic acid and arabinose or galactose or both arabinose and galactose, and removing the solution, thereby obtaining, sperm with improved function.

4. The method of claim **2** wherein the solution comprises said polymer.

5. A method for extending sperm to obtain sperm with improved function, comprising contracting a sample containing sperm with a solution comprising a hexuronic acid monomer or with a solution comprising a polymer wherein said polymer comprises hexuronic acid and arabinose or galactose or both arabinose and galactose, thereby obtaining sperm with improved function.

6. The method of claim **5** wherein the solution comprises said polymer.

7. A sperm wash medium comprising a solution comprising a balanced salt solution and a hexuronic acid monomer or a solution comprising a balanced salt solution and a polymer wherein said polymer comprises a hexuronic acid and arabinose or galactose or both arabinose and galactose, at a concentration sufficient to improve sperm function.

8. The medium of claim **7** wherein the solution comprises a balanced salt solution and said polymer.

9. A sperm isolation medium for isolation of sperm by centrifugation, comprising a solution comprising a density gradient material and a hexuronic acid monomer or a solution comprising a density gradient material and a polymer wherein said polymer comprises hexuronic acid and arabinose or galactose or both arabinose and galactose.

10. The medium of claim **9** wherein the solution comprises a density gradient material and said polymer.

11. A sperm isolation medium for the isolation of sperm by swim-up method, comprising a solution comprising a macromolecule and a hexuronic acid monomer or a solution comprising a macromolecule and a polymer wherein said polymer comprises hexuronic acid and arabinose or galactose or both arabinose and galactose.

12. The medium of claim **11** wherein the solution comprises a macromolecule and said polymer.

13. An extending or culturing medium comprising a solution comprising a balanced salt solution and a hexuronic acid monomer or a solution comprising a balanced salt solution and a polymer wherein said polymer comprises hexuronic acid and arabinose or galactose or both arabinose and galactose, at a concentration sufficient to improve sperm, oocyte, embryo, or embryonic stem cell (ESC) function.

14. The medium of claim **13** wherein the solution comprises a balanced salt solution and said polymer.

15. A method for reducing loss of functional sperm during storage of sperm in a refrigerated, frozen or vitrified state, comprising the steps of:

(a) combining a hexuronic acid monomer, or combining a polymer wherein said polymer comprises hexuronic acid and arabinose or galactose or both arabinose and galactose, with a sample containing sperm, wherein said hexuronic acid monomer or said polymer is in an amount effective to reduce said loss; and

(b) storing said sample in a refrigerated, frozen or vitrified state.

16. The method of claim **15** wherein in step (a) said polymer is combined with a sample containing sperm.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,140,121
DATED : October 31, 2000
INVENTOR(S) : Joanna E. Ellington and Sylvia Adams Oliver

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 31,
Line 15, "solution, thereby obtaining, sperm" should read -- solution, thereby obtaining sperm --.
Line 16, "The method of claim 2" should read -- The method of Claim 3 --.

Signed and Sealed this

Fifteenth Day of April, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

RALPH
MCELROY TRANSLATION
COMPANY

May 27, 2004

Re: RMTC Job No. 1604-98342

To Whom It May Concern:

This is to certify that a professional translator on our staff who is skilled in the **German** language translated the document(s) noted below from **German** into **English**.

- German Article (Deep Freezing of Rabbit Sperm)

We certify that the attached **English** translation conforms essentially to the original **German** language.

Kim Vitray
Operations Manager

Subscribed and sworn to before me this 27TH day of MAY 2004.

TINA WUELFING
Notary Public
State of Texas
My Commission Expires
December 8, 2007

Tina Wuelfing
Notary Public

910 WEST AVE.
AUSTIN, TEXAS 78701
www.mcelroytranslation.com

(512) 472-6753
1-800-531-9977
FAX (512) 472-4591

C. Hellemann[1] and E. Gigoux[1]: Deep Freezing of Rabbit Sperm. Effect of a Surfactant on Fertilizing Capacity[2]

Zuchthyg., 23, 33-37 (1988)

1 Center for Artificial Insemination, Southern University of Chile, Valdivia, Chile

2 Received date: May 22, 1987

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910 West Avenue, Austin, Texas 78701 USA

DEEP FREEZING OF RABBIT SPERM
EFFECT OF A SURFACTANT ON FERTILIZING CAPACITY

In order to clear, if the protective effect of a Na-lauryl-sulphat containing product (OEP) on acrosomes would increase the fertilizing capacity of frozen rabbit semen, 19 ejaculates were frozen after dilution with TRIS-extenders containing 4,5% (fraction I), 9,0% (fraction III) dimethyl-sulphoxyde (DMSO) without and with 0,2 % OEP (fractions II and IV). Semen was frozen in 0,25 straws. OEP did not have any effect on motility (MOT) of thawed semen, but had a significant effect on acrosome integrity (NAR), compairing both 9,0 % DMSO fractions. 50 does were inseminated with each frozen semen fraction and another 50 with fresh semen as controls (fraction V). Conception rates (I=42%, II=34.7%, III=18%, IV=28%, V=52%) and litter size (I=6.0, II=4.8, III=3.3, IV=2.5, V=6.2) indicate that the protective effect of OEP on acrosomes does not or only conditionally increases the conception-rates. A negative effect of OEP on fertilizing capacity of sperm could not be stated.

The importance of the evolution of the acrosomes as a spermatological parameter, which was first incorporated by Weitze et al. (1975), was corroborated in continuing experiments to the extent that there is a significant relationship between the fraction of normal acrosomes in deep frozen (DF) sperm and the number of fetuses born per insemination (Helleman, 1976), or that a minimum number of "intact" spermatozoa in the insemination dose is necessary for undisturbed fertilization (Weitze, 1977; Weitze 1981). Weitze, et al. (1975) called attention to the acrosome-damaging effect of the cryoprotector dimethylsulfoxide (DMSO) in the deep freezing of sperm, according to comparative tests with end concentrations of 2.5% and 4.5% DMSO in the semen extender. This effect was confirmed in comparative tests of extenders containing 2%, 4.5%, 7% and 9% DMSO, where a clearly elevated number of damaged acrosomes was found, in particular with 9% DMSO in comparison to 2% DMSO (Hellemann, 1976). However, in the same tests, the acrosome damage could be significantly reduced through the addition of 0.2% OEP*, a surface-active detergent. However, a clear effect on the insemination results could not be detected in a preliminary insemination experiment. The goal of this work was to investigate the effect of added OEP on the spermatological parameter motility (MOT) and the acrosome integrity in expanded deep freezing tests and to verify in an insemination test whether the fertilizing capacity of the DF sperm can be increased through the addition of OEP.

Material and methods

The semen donors were 9 Angora bucks, which were used at an insemination station to provide the insemination service with fresh sperm. The test inseminations were carried out in

* OPE = "Orvus ES Paste." Na lauryl sulfate and other organic components. New name: "EQUEX STM" Nova Chemical Sales, Inc., P. O. Box 144, Scituate, Mass. 02066.

commercial angora breeding operations. There were 19 ejaculates available for processing, which were divided into 4 fractions and diluted at room temperature as single phase to about 3 to 5 million sperm cells per dose with the extenders indicated in Table 1, filled into 0.25-mL straws and packed in aluminum round cassettes, and then, after 2 hours adaptation time, frozen at +5°C in nitrogen vapor 2 cm above the nitrogen level. One thawed sample per ejaculate was tested for motility (MOT) and, after being fixed with a physiological salt solution containing 0.3% NaF, the fraction of undamaged acrosomes was counted by contrast microscope evaluation of the normal apical edge (NAE). Of the deep frozen ejaculates, 4 were used for the test inseminations. 250 animals were inseminated, alternating one doe per fraction in each case, followed by a 5th control insemination with fresh sperm. To initiate ovulation 0.2 mL of the synthetic GnRH preparation Receptal® was injected i.m. immediately after each insemination.

Table 1: Composition of extenders comparatively used for freezing rabbit semen

(1) FRAKTION	1	2	3	4
Stammlösung* (2)	74,0 ml	74,0 ml	74,0 ml	74,0 ml
DMSO	4,5 ml	4,5 ml	9,0 ml	9,0 ml
OEP	–	0,2 ml	–	0,2 ml
Aqua dest.	4,5 ml	4,5 ml	–	–
(3) Eigelb	17,0 ml	17,0 ml	17,0 ml	17,0 ml

* Stammlösung: (2)	
(4) TRIS (hydroxymethyl)-aminomethan	2,523 g
DGlucose	1,042 g
Citronensäure anh. (5)	1,276 g
Aqua dest. ad.	75,5 ml
(6) Glycerin	0,5 ml

[Editor's note: In figures and tables, commas in numbers represent decimal points.]

Key:
1 Fraction
2 Parent solution
3 Egg yolk
4 Tris(hydroxymethyl)aminomethane
5 Anhydrous citric acid
6 Glycerol

The measured values of the spermatological parameters MOT and NAE were graphically plotted as the accumulated polygon curve of the frequency distribution (Lorenz, 1984) and the test differences were statistically verified by the Kruskal-Wallis test. Conception rates, litter size and number of fetuses born were subjected to chi^2 tests. In all cases, an error probability of 5% applied.

Results

a) Effect of OEP on the parameters MOT and NAE.

The distribution of the measurement MOT values of the 19 frozen ejaculates can be seen in Figure 1. The MOT values represented there did not show significant differences between the extenders that were used. It is also evident that, in 70-80% of the samples, tested the estimated MOT values lay chiefly between 10 and 30%.

The distribution of the measured NAE values of the same ejaculates follows in Figure 2. From this, one can see that, in the case of the 9.0% DMSO fraction, about 40% of the samples showed under 5% NAE and none were over 20%. The difference to the 3 comparison fractions is significant.

b) Effect of OEP on the fertilization parameters

The results of the test inseminations are given in Table 2. This table shows that the 4.5% DMSO fraction came closest to the fresh sperm fraction both with regard to the conception rate and the litter size. In the case of the 9.0% DMSO fractions, the litter sizes and animals born per insemination were significantly lower than those for the fresh sperm fraction, and in some cases even the 4.5% DMSO fraction.

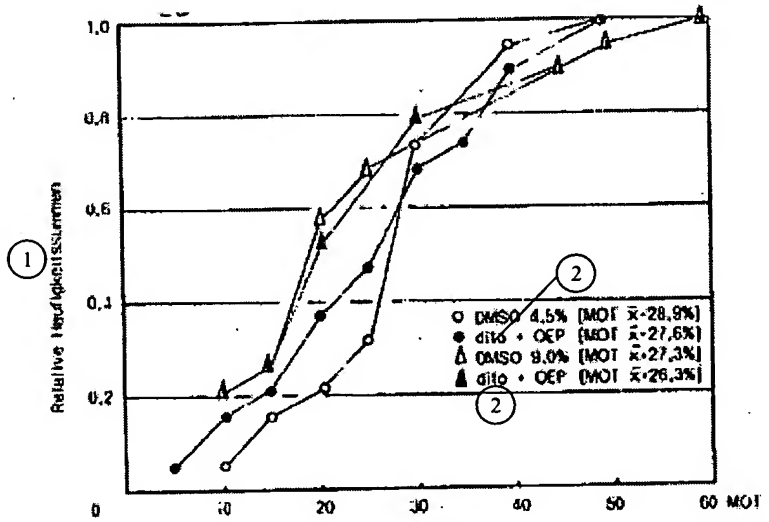

Fig. 1: Accumulated distribution of motility-rates (MOT) in rabbit semen frozen with extenders containing 4.5% and 9.0% dimethyl-sulphoxide (DMSO), comparatively added with 0,2% OEP (n=19).

Key: 1 Relative frequency totals
2 Ditto

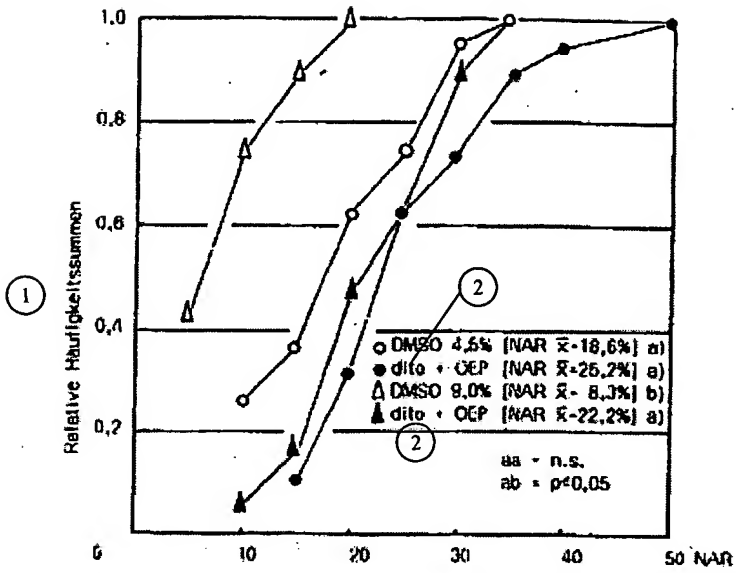

Fig. 2: Accumulated distribution of spermatozoa with normal acrosomal ridge (NAR) in rabbit semen frozen with extenders containing 4.5% and 9.0% dimethyl-sulphoxide (DMSO), comparatively added with 0.2% OEP (n=19).

Key: 1 Relative frequency totals
2 Ditto

Table 2: Conception-rates, litter size and young born by insemination with fresh and frozen rabbit semen containing 4,5% and 9,0% dimithyl-sulphoxide (DMSO), comparatively added with 0,2% OEP

(1) FRAKTION	I 4,5% DMSO	II 4,5% DMSO +0,2% OEP	III 9,0% DMSO	IV 9,0% DMSO +0,2% OEP	V
		(2) TG-SPERMA			(3) FRISCH-SPERMA
(4) Besamungen (n)	50	49	50	50	50
Konzeptionsrate (%)	42,0 a	34,7 a	18,0 b	28,0 a	52,0 a
Wurfgröße ($\bar{X}$)	6,0 a	4,8	3,3	2,5 b	6,2 a
Früchte/Besamung	2,5 a	1,7	0,3 b	0,7 c	3,2 a

(5) aa = nicht signifikant
ab = $p \leq 0,05$
ac = $p \leq 0,05$

Key: 1 Fraction
2 DF sperm
3 Fresh sperm
4 Inseminations
Conception rate
Litter size
Fetuses/insemination
5 aa = not significant

Discussion

The expected motility differences were not detected in the comparison of the DMSO concentrations of 4.5% and 9.0%, in contrast to earlier studies, where a tendency toward an elevation of motility with increasing DMSO concentration was evident in a comparison of extender containing 2.0%, 4.5%, 7.0% and 9.0% DMSO (Helleman, 1976) (Figure 1). One can also see from Figure 1 that the addition of OEP did not have any effect on motility. In contrast, one can clearly see a distinct protective effect on the acrosome integrity by OEP, which produced a significant difference between the 9.0% DMSO fractions (Figure 2), where the NAE values for the 9.0% DMSO fraction + OEP reached approximately the same level as the other fractions.

Thus, according to the microscope tests, the spermatological parameters MOT and NAE for fractions I, II and IV were nearly comparable, and, as expected, would probably also produce comparable fertilization results. However, it can be seen from the evaluation of the fertilization parameters in Table 2 that the increase of the NAE values produced by OEP were not or were only conditionally accompanied by an elevation of the fertilization results. Only in the comparison of the two 9.0% DMSO fractions could the higher conception rate be ascribed to the effect of OEP. The addition of OEP to the 4.5% DMSO fraction did produce lower values in the conception rate and litter size, but the deviations could not be statistically confirmed.

All in all, the achieved conception rates appear to be rather moderate, even for the control inseminations with fresh sperm. However, these achieved similar percentages as the approximately 2000 routine inseminations conducted in the same time period, late autumn 1986, using fresh sperm. Coats that were too long for the reproduction performance at the time of the insemination, as already described by Brockhausen et al. (1985), and day/night light ratios not matched to the season in the cages (Lange, 1984) of most breeding operations were found and probably contributed as factors to the less than completely satisfactory results. Finally, it can be concluded from the results that the acrosome-protecting effect of OEP is not or is only conditionally accompanied by an increase of the fertilization capacity of the sperm. A reduction of the fertilization capacity of the sperm produced by the addition of OEP could not be statistically confirmed.

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EFFECTS OF EQUEX STM PASTE ON VIABILITY OF FROZEN-THAWED DOG SPERMATOZOA DURING IN VITRO INCUBATION AT 38°C

A. Rota, B. Ström, C. Linde-Forsberg[1] and H. Rodriguez-Martinez

Department of Obstetrics and Gynecology
Faculty of Veterinary Medicine
Swedish University of Agricultural Sciences
Box 7039, 750 07 Uppsala, Sweden

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ABSTRACT

In the canine, artificial insemination with cryopreserved semen generally yields lower pregnancy rates with vaginal deposition than with uterine deposition, one of the reasons being the shortened life span of frozen-thawed spermatozoa. The incubation of spermatozoa at body temperature partially mimics the situation in vivo, and evaluation of the kinetics of viability loss under these conditions can be used to measure the damage caused by freezing and thawing procedures. In this study, 2 aliquots were separated from split ejaculates collected from 7 dogs and were frozen by lowering the straws, in 3 steps, into an LN_2 tank after dilution with egg yolk Tris-citrate-glucose extender with or without the addition of 0.5% Equex STM paste. Motility and plasma membrane integrity (evaluated with the combined fluorescent probes 6-carboxyfluorescein diacetate and propidium iodide) were assessed immediately after thawing and over the next 3 h at 38°C. The addition of Equex STM paste significantly increased the proportion of spermatozoa having an intact plasmalemma immediately after thawing compared with the control. It also increased the longevity of the thawed spermatozoa, prolonging the maintenance of both motility and plasma membrane integrity.

Key words: dog, frozen semen, sodium lauryl sulphate, membrane integrity

INTRODUCTION

Higher pregnancy rates using frozen-thawed semen are obtained in the canine with intrauterine than with intravaginal insemination (9,10,20,26). Semen deposition in the uterus can be achieved by laparotomy or laparoscopy (20,25,26), or by cervical catheterization (2,15,31), a technique which is less stressful for the bitch but which requires much training. One reason for the lower

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[1]Author to whom reprint requests should be addressed.

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fertility after vaginal deposition is considered to be a shortened life span of frozen-thawed spermatozoa. This is supported by the need for optimal timing of insemination when using cryopreserved spermatozoa (16).

Many extenders have been studied for canine semen cryopreservation. Tris-based extenders are commonly used in both practical and experimental situations and are known to give good pregnancy rates (2,9,19). Compounds containing sodium dodecyl (lauryl) sulphate (SDS) have been included in extenders for several species; SDS is a water-soluble anionic detergent and wetting agent, used to solubilize proteins. The addition of different amounts of Equex STM paste or Orvus ES paste, both containing SDS, to extenders for the freezing of semen has been found beneficial in the boar (22), bull (3), stallion (18) and mouse (21), enhancing motility (3,18,22) and acrosome integrity (3,22) and giving high fertilization rates both in vivo (3,18) and in vitro (21). Orvus ES paste has also been associated with increased longevity of dog spermatozoa at 22°C (29). High pregnancy rates (over 85%) were obtained in the canine with intravaginal deposition of semen, that had been cryopreserved in an extender containing Equex STM paste (19).

Incubation in vitro of frozen-thawed spermatozoa at body temperature partially mimics the situation in vivo, and repeated evaluations of motility under this condition have been used to test semen quality in various species. Low post-thaw maintenance of motility during incubation at 37 to 39°C is a known phenomenon in frozen canine semen (8,20,28), perhaps more so than for semen from other species (5). Whether or not the addition of SDS to the extender increases the longevity of frozen-thawed dog spermatozoa during incubation at body temperature has not been studied.

The aim of the present study was to observe the effects of adding Equex STM paste to an extender for the freezing of dog semen on in vitro post-thaw viability during incubation at 38°C, as expressed by motility and plasma membrane integrity.

MATERIALS AND METHODS

Animals

The dogs included in this study were 2 Rottweilers, 1 Bavarian Schweisshund, 2 Beagles, 1 German Shepherd and 1 crossbreed, all privately owned. Their ages ranged between 2 and 8 yr. For the ejaculates to be included in the study, motility and the percentage of morphologically normal spermatozoa had to be ≥ 70%.

Diluents and Extenders

A Tris diluent (TRIS buffer), containing 2.4 g Tris, 1.4 g citric acid, 0.8 g glucose, 0.06 g Na-benzylpenicillin and 0.1 g streptomycin sulphate solubilized in 100 ml of distilled water, was prepared in one batch and stored frozen (pH 6.60, 253 mOsm). This buffer was used for the dilution 1:3 of the samples of fresh and equilibrated semen prior to evaluation and of the cryopreserved semen immediately after thawing.

Each freezing extender (Table 1; 20,29) was prepared as a single batch and was stored frozen. Extender 1 was used for semen dilution prior to equilibration, while either Extender 2 or Extender 2-E was added prior to freezing.

Table 1. Composition of freezing extenders

	Extender 1	Extender 2	Extender 2-E
Tris	2.4 g	2.4 g	2.4 g
Citric acid, monohydrate	1.4 g	1.4 g	1.4 g
Glucose	0.8 g	0.8 g	0.8 g
Na-benzylpenicillin	0.06 g	0.06 g	0.06 g
Streptomycin sulphate	0.1 g	0.1 g	0.1 g
Egg yolk	20 ml	20 ml	20 ml
Glycerol	3 ml	7 ml	7 ml
Equex STM paste[a]	-	-	1 ml
Distilled water	to 100 ml	to 100 ml	to 100 ml
pH	6.53	6.56	6.48
Osmolarity	740 mOsm	1390 mOsm	1370 mOsm

[a]Nova Chemical Sales, Scituate, Inc., MA, USA.

The 2 treatments differed only in the composition of the extenders: Semen diluted with Extender 1 plus Extender 2 is referred to as Egg Yolk Tris-Glycerol (EYT-G), while that diluted with Extender 1 plus Extender 2-E is called Egg Yolk Tris-Glycerol-Equex (EYT-GE).

Semen Evaluation

Morphology. Smears of the fresh, undiluted semen were made for sperm head morphology assessment (stained with carbol-fuchsine stain; 30) and for the detection of cells other than spermatozoa (Papanicolaou's stain, Histolab Products AB, Västra Frölunda, Sweden). An aliquot was fixed in buffered formol-saline (4) to be evaluated for tail, midpiece and acrosome defects.

Motility. Motility was assessed subjectively using a phase contrast microscope at 37°C and x 400 magnification. Two aliquots from each semen sample were placed on a glass slide under a coverslip, and at least 8 fields were examined in each subsample.

Plasma membrane integrity. Sperm membrane integrity was assessed using 6-carboxyfluorescein diacetate and propidium iodide (C-FDA/PI) (11, modified; 23). Semen (50 µl) was diluted with staining medium (150 µl) and incubated in the dark for 15 to 25 min at 30°C. A 5-µl aliquot of stained suspension was placed on a slide and covered with a coverslip, and the fields were observed at x 400 magnification with epifluorescence UV-illumination on a Diaplan Leitz microscope. For each sample stained with C-FDA/PI, 100 sperm cells were counted in 2 slides (total=200) and classified as Group G - having an intact plasmalemma when stained green with C-FDA and unstained with PI; Group GR - having a damaged plasmalemma but an intact acrosome when the acrosome stained green with C-FDA but the post-acrosomal region stained red with PI; and Group R - having both a damaged plasmalemma and a damaged acrosomal membrane when the cells remained unstained with C-FDA but stained red with PI.

Experimental Design

One ejaculate was obtained from each dog. After taking a sample of fresh semen for evaluation, 2 aliquots were separated from each ejaculate. One was diluted with EYT-G and the other with EYT-GE; both were then equilibrated and frozen together in a single operation. Samples of fresh, equilibrated and cryopreserved semen, the latter analyzed immediately after thawing and after 1, 2 and 3 h of incubation at 38°C, were evaluated for motility and plasma membrane integrity.

Semen Collection

The sperm-rich fraction of 1 ejaculate was collected from each dog in a calibrated plastic vial by digital manipulation (15). The volume was measured and the sperm concentration determined with a photometer (SpermaCue, Minitüb, Tiefenbach, Germany). Before being assessed for motility and plasma membrane integrity, the samples were diluted 1:3 in TRIS buffer.

Semen Dilution and Freezing

Two aliquots of semen containing $150x10^6$ spermatozoa each were placed in each of 2 plastic vials and centrifuged at 700g for 6 min. The supernatant was discarded from both samples, and the pellets were diluted in 2 steps with the freezing extenders (Table 1). First, 1.5 ml of Extender 1 was added at room temperature, and the extended semen was placed in a cooler, where the temperature in the sample reached 4°C in about 45 min. One hour after beginning the cooling procedure, 1.5 ml of Extender 2 or Extender 2-E, also at 4°C, was added and mixed by gently reversing the tubes several times. The final sperm concentration was $50x10^6$/ml. After equilibration, an aliquot from each treatment was separated, diluted 1:3 in TRIS buffer and immediately assessed for motility and plasma membrane integrity.

At 75 min from the onset of cooling, the extended semen was frozen in 0.5-ml straws, 4 in each of 2 goblets, by lowering them, in 3 steps, in an Apollo SX-18 LN_2 tank (MVE Cryogenetics®, New Prague, Minn. USA) containing 15 to 18 cm of LN_2 for 2, 2 and 1 min, with the goblets on top of the canes and the top of the goblets at 7, 13 and 20 cm below the opening of the tank. (10, modified, 28). Before introducing the canes and the start of freezing, the empty canister was kept in the first position for 5 min. Just before freezing, an aliquot of extended and equilibrated semen from each sample was diluted 1:3 in TRIS buffer and was assessed both for motility and plasma membrane integrity.

Semen Thawing and Incubation

Two straws for each extender and dog were thawed in a waterbath at 38°C for 1 min and emptied into a tube. A 200-µl portion of thawed semen was then placed in a 3-ml plastic tube, and 400 µl of TRIS buffer, also at the same temperature, was slowly added. The semen was placed in the closed vials in the dark at 38°C and held there for 3 h. Samples were evaluated for motility and plasma membrane integrity immediately after dilution and at each hour for up to 3 h during the incubation at 38°C.

Statistical Analysis

The mean values obtained for each dog were used to compare the extenders, using the Wilcoxon matched pairs signed rank sum test. All analyses were performed using Minitab Statistical Software (Minitab Inc., State College, PA, USA). Values are presented as mean ± standard deviation, and were considered statistically significant when $P<0.05$.

RESULTS

Neither the extender nor the equilibration had any significant effect on motility and plasma membrane status (Group G, Group GR, Group R) when fresh semen and equilibrated samples (equilibrated EYT-G and equilibrated EYT-GE) were compared.

The motility and plasma membrane status of the spermatozoa in the fresh and diluted semen after equilibration are summarized in Table 2.

Table 2. Motility and proportion of spermatozoa (%) with intact plasma membrane (Group G), damaged plasmalemma but intact acrosome (Group GR), and damaged plasmalemma and acrosome (Group R) estimated using the fluorophores 6-carboxyfluorescein diacetate/propidium iodide (C-FDA/PI) prior to freezing in the fresh semen and in semen after equilibration for the 2 treatments. Mean ± SD (n = 7)

	Fresh semen	Equilibrated EYT-G	Equilibrated EYT-GE
Motility	82.1±6.4	82.9±2.7	82.1±3.9
C-FDA/PI Group G	94.5±2.8	92.0±3.1	92.8±2.8
C-FDA/PI Group GR	2.3±1.1	2.9±0.9	3.1±1.5
C-FDA/PI Group R	3.2±3.0	5.1±2.5	4.1±1.5

The extender had no significant effect on motility immediately after thawing ($P=0.093$), but the difference was significant after 1, 2 ($P<0.03$) and 3 ($P<0.05$) h of incubation, with motility being consistently higher in EYT-GE. The mean relative decrease in motility from Hour 0 to Hour 1 was 19.4% ±13.2 for EYT-GE and 65.3% ±26.5 for EYT-G ($P<0.03$). The corresponding decrease from Hour 0 to Hour 3 was 42.3% ±30.9 for EYT-GE and 87.6% ±11.4 for EYT-G ($P<0.05$).

The effect of the extender on the percentage of spermatozoa belonging to Group G was significant immediately after thawing ($P<0.05$) and after 1, 2 and 3 h of incubation at 38°C ($P<0.03$). The proportion of spermatozoa belonging to Group GR was always significantly ($P<0.03$) higher in EYT-GE, while the proportion of spermatozoa belonging to Group R was always significantly ($P<0.03$) higher when the semen was frozen in EYT-G. The mean relative decrease in C-FDA/PI Group G from Hour 0 to Hour 1 was 22.4% ±9.0 for EYT-GE and 67.8% ±10.4 for EYT-G. The corresponding decrease from Hour 0 to Hour 3 was 23.7% ±8.4 for EYT-GE and 72.9% ±13.9 for EYT-G, the effect of the extender being significant ($P<0.03$) on both occasions.

Values for motility and plasma membrane status in the cryopreserved semen immediately after thawing and during incubation at 38°C are shown in Table 3.

Table 3. Motility and proportion of spermatozoa (%) with intact plasma membrane (Group G), damaged plasmalemma but intact acrosome (Group GR), and damaged plasmalemma and acrosome (Group R) estimated using the fluorophores 6-carboxyfluorescein diacetate/propidium iodide (C-FDA/PI) in the frozen-thawed semen in the 2 treatments, immediately after thawing and after incubation at 38°C for 1, 2 and 3 hours. Mean ± SD (n = 7)

EYT-G Hours post thawing:	0	1	2	3
Motility	56.4±5.6	20.0±17.3	9.6±9.2	7.1±7.1
C-FDA/PI group G	56.5±12.8	18.2±8.2	16.7±12.2	16.2±11.3
C-FDA/PI group GR	1.2±1.6	0.6±0.4	0.7±0.8	0.7±0.8
C-FDA/PI group R	41.5±11.3	81.3±8.1	82.9±12.0	82.7±11.3
EYT-GE Hours post thawing:	0	1	2	3
Motility	61.4±7.7	50.0±12.6	41.8±18.1	35.4±19.5
C-FDA/PI group G	63.0±9.6	48.7±9.0	45.9±6.9	48.1±9.6
C-FDA/PI group GR	4.7±1.7	4.7±1.0	4.9±1.9	4.6±1.1
C-FDA/PI group R	32.4±8.9	46.6±9.0	49.0±7.3	47.3±9.7

DISCUSSION

The present results clearly demonstrate that the addition of Equex STM Paste is beneficial for freezing dog semen. Both viability and longevity post-thaw were enhanced, as had been observed in the findings of Thomas et al. (1992). The active compound in Equex STM Paste is thought to be the detergent SDS, which probably exerts its action through the alteration of the egg yolk contained in the extender (22). The interaction with the egg yolk could involve the solubilization of active molecules. This hypothesis is supported by the observation that when only the supernatant was used in preparing the extenders, SDS had a stronger positive effect on post-thaw motility when added to the egg yolk before centrifugation than when added later (21). Although a direct positive effect of SDS on the plasma membrane has also been hypothezised, Pursel et al. (22) found reduced sperm viability when SDS was added to extenders depleted of egg yolk, thus indicating the contrary. Moreover, detergents by themselves, including SDS, are known to have a spermicidal effect (17).

To evaluate preservation techniques, various quality traits of cryopreserved semen can be investigated. The most common viability tests are the subjective evaluation of motility and progressive motility, despite the poor repeatability between observers. More objective measurements of motility, such as with computer-assisted sperm analyzers are available, but their use is not always free of bias (6). Plasma membrane integrity and function are essential for cell viability, as the selective permeability of the plasma membrane maintains intracellular metabolic activities, pH and ionic composition. In the sperm cell, a functional plasma membrane is also essential for the events leading to fusion with the oocyte (7). In our study, the combination of 2 fluorophore probes (C-FDA and PI), already used to evaluate the plasma membrane integrity of spermatozoa in the canine (23,28) and in other species (e.g. 11), was used as a more objective measurement of viability.

To partially mimic the in vivo situation, the spermatozoa were incubated at 38°C after thawing. Although the environment in vitro is clearly different from the one in vivo, repeated motility estimates during incubation at 37 to 38°C have been found to give a better indication of

fertility than the estimation of immediate post-thaw motility in the boar (13) and the bull (24). Motility assessment made after incubation of fresh semen for 4 or 24 h under similar conditions were effective in identifying men with poor IVF and pregnancy outcome following assisted reproduction techniques (1,27). In the canine, motility estimates after incubation at body temperature have been used in the evaluation of different freezing techniques (8,20,28). England (8), who monitored the proportions of motile and viable dog spermatozoa (negrosin/eosin stain) during a 6-h period, found that in fresh semen samples diluted in a Tes/Tris buffer and incubated at 39°C, 75% of the initial motility was lost in about 7 h, whereas after cryopreservation post-thaw motility and viability decreased to the same extent within 3 h. In the present study, only 42% of motility and 24% of viable cells were lost after 3 h of incubation, when Equex STM paste was added to the extender.

Immediately after thawing, the proportion of spermatozoa with an intact plasma membrane was higher when Equex STM Paste was added to the freezing extender. Although a tendency was also seen at Hour 0 for motility to be higher when using Equex STM Paste, the difference was not significant (P=0.093). Thus, immediately after thawing, the C-FDA/PI staining technique seemed to be a better predictor of longevity of the spermatozoa than the subjective evaluation of motility.

After the decrease in the percentage of spermatozoa showing an intact plasma membrane during the initial hour after thawing, the mean proportion of spermatozoa belonging to Group G was fairly constant from the first to the third hour of incubation. The absence of further decrease after the first hour after thawing was seen in both treatments. Thus the decrease in the percentage of intact spermatozoa during the incubation at body temperature seems to reflect latent damage caused by freezing or thawing, rather than an ageing-related decline. The addition of this measure to the immediate post-thaw evaluation could therefore help in assessing the effects of the different techniques used for cryopreservation. By contrast, the decrease in motility continued throughout the 3 h of incubation, and the proportion of sperm cells with an intact plasma membrane was generally higher than the proportion of motile sperm cells, as previously described in the bull by Januskauskas and Rodriguez-Martinez (12). It is not known whether these spermatozoa can regain their motility and fertilizing capacity in the female genital tract, but it has been shown in the boar that some immotile spermatozoa can become motile after appropriate stimuli, e.g. with caffeine (14).

The freezing technique used in this experiment is quick and easy, since neither long equilibration periods nor complex or expensive freezing equipment is needed. Furthermore, it showed promising results in vitro. A similar freezing technique, but one that uses other extenders and another thawing medium, is the CLONE system, with reported pregnancy rates above 85% after intrauterine AI (10). Sperm motility during incubation at body temperature seems to be maintained longer with the extender used in our study than with CLONE, although we cannot speculate on the role of the thawing medium used in the CLONE method on both the in vitro viability and the obtained pregnancy rates (10,28). Intravaginal insemination with semen frozen using a different freezing technique but in an extender containing Equex STM Paste resulted in an overall pregnancy rate of 87.5% (35/40), similar to that obtained after natural mating (19). In vitro evaluation of sperm recovery and longevity after the freezing procedure used for these inseminations have not been published.

In conclusion, the results of this study indicate that the addition of Equex STM paste to a Tris extender for dog semen freezing improves both post-thaw viability and longevity of the

cryopreserved spermatozoa during incubation at body temperature, as judged by motility and plasmalemma integrity. Further studies are needed to evaluate fertility after AI using cryopreserved semen processed with this freezing technique.

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Syllabus

NOTE: Where it is feasible, a syllabus (headnote) will be released, as is being done in connection with this case, at the time the opinion is issued. The syllabus constitutes no part of the opinion of the Court but has been prepared by the Reporter of Decisions for the convenience of the reader. See *United States* v. *Detroit Timber & Lumber Co.*, 200 U. S. 321, 337.

SUPREME COURT OF THE UNITED STATES

Syllabus

KSR INTERNATIONAL CO. *v.* TELEFLEX INC. ET AL.

CERTIORARI TO THE UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

No. 04–1350. Argued November 28, 2006—Decided April 30, 2007

To control a conventional automobile's speed, the driver depresses or releases the gas pedal, which interacts with the throttle via a cable or other mechanical link. Because the pedal's position in the footwell normally cannot be adjusted, a driver wishing to be closer or farther from it must either reposition himself in the seat or move the seat, both of which can be imperfect solutions for smaller drivers in cars with deep footwells. This prompted inventors to design and patent pedals that could be adjusted to change their locations. The Asano patent reveals a support structure whereby, when the pedal location is adjusted, one of the pedal's pivot points stays fixed. Asano is also designed so that the force necessary to depress the pedal is the same regardless of location adjustments. The Redding patent reveals a different, sliding mechanism where both the pedal and the pivot point are adjusted.

In newer cars, computer-controlled throttles do not operate through force transferred from the pedal by a mechanical link, but open and close valves in response to electronic signals. For the computer to know what is happening with the pedal, an electronic sensor must translate the mechanical operation into digital data. Inventors had obtained a number of patents for such sensors. The so-called '936 patent taught that it was preferable to detect the pedal's position in the pedal mechanism, not in the engine, so the patent disclosed a pedal with an electronic sensor on a pivot point in the pedal assembly. The Smith patent taught that to prevent the wires connecting the sensor to the computer from chafing and wearing out, the sensor should be put on a fixed part of the pedal assembly rather than in or on the pedal's footpad. Inventors had also patented self-contained modular sensors, which can be taken off the shelf and attached to any

mechanical pedal to allow it to function with a computer-controlled throttle. The '068 patent disclosed one such sensor. Chevrolet also manufactured trucks using modular sensors attached to the pedal support bracket, adjacent to the pedal and engaged with the pivot shaft about which the pedal rotates. Other patents disclose electronic sensors attached to adjustable pedal assemblies. For example, the Rixon patent locates the sensor in the pedal footpad, but is known for wire chafing.

After petitioner KSR developed an adjustable pedal system for cars with cable-actuated throttles and obtained its '976 patent for the design, General Motors Corporation (GMC) chose KSR to supply adjustable pedal systems for trucks using computer-controlled throttles. To make the '976 pedal compatible with the trucks, KSR added a modular sensor to its design. Respondents (Teleflex) hold the exclusive license for the Engelgau patent, claim 4 of which discloses a position-adjustable pedal assembly with an electronic pedal position sensor attached a fixed pivot point. Despite having denied a similar, broader claim, the U. S. Patent and Trademark Office (PTO) had allowed claim 4 because it included the limitation of a fixed pivot position, which distinguished the design from Redding's. Asano was neither included among the Engelgau patent's prior art references nor mentioned in the patent's prosecution, and the PTO did not have before it an adjustable pedal with a fixed pivot point. After learning of KSR's design for GMC, Teleflex sued for infringement, asserting that KSR's pedal system infringed the Engelgau patent's claim 4. KSR countered that claim 4 was invalid under §103 of the Patent Act, which forbids issuance of a patent when "the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art."

Graham v. *John Deere Co. of Kansas City*, 383 U. S. 1, 17–18, set out an objective analysis for applying §103: "[T]he scope and content of the prior art are . . . determined; differences between the prior art and the claims at issue are . . . ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented." While the sequence of these questions might be reordered in any particular case, the factors define the controlling inquiry. However, seeking to resolve the obviousness question with more uniformity and consistency, the Federal Circuit has employed a "teaching, suggestion, or motivation" (TSM) test, under which a pat-

ent claim is only proved obvious if the prior art, the problem's nature, or the knowledge of a person having ordinary skill in the art reveals some motivation or suggestion to combine the prior art teachings.

The District Court granted KSR summary judgment. After reviewing pedal design history, the Engelgau patent's scope, and the relevant prior art, the court considered claim 4's validity, applying *Graham*'s framework to determine whether under summary-judgment standards KSR had demonstrated that claim 4 was obvious. The court found "little difference" between the prior art's teachings and claim 4: Asano taught everything contained in the claim except using a sensor to detect the pedal's position and transmit it to a computer controlling the throttle. That additional aspect was revealed in, *e.g.*, the '068 patent and Chevrolet's sensors. The court then held that KSR satisfied the TSM test, reasoning (1) the state of the industry would lead inevitably to combinations of electronic sensors and adjustable pedals, (2) Rixon provided the basis for these developments, and (3) Smith taught a solution to Rixon's chafing problems by positioning the sensor on the pedal's fixed structure, which could lead to the combination of a pedal like Asano with a pedal position sensor.

Reversing, the Federal Circuit ruled the District Court had not applied the TSM test strictly enough, having failed to make findings as to the specific understanding or principle within a skilled artisan's knowledge that would have motivated one with no knowledge of the invention to attach an electronic control to the Asano assembly's support bracket. The Court of Appeals held that the District Court's recourse to the nature of the problem to be solved was insufficient because, unless the prior art references addressed the precise problem that the patentee was trying to solve, the problem would not motivate an inventor to look at those references. The appeals court found that the Asano pedal was designed to ensure that the force required to depress the pedal is the same no matter how the pedal is adjusted, whereas Engelgau sought to provide a simpler, smaller, cheaper adjustable electronic pedal. The Rixon pedal, said the court, suffered from chafing but was not designed to solve that problem and taught nothing helpful to Engelgau's purpose. Smith, in turn, did not relate to adjustable pedals and did not necessarily go to the issue of motivation to attach the electronic control on the pedal assembly's support bracket. So interpreted, the court held, the patents would not have led a person of ordinary skill to put a sensor on an Asano-like pedal. That it might have been obvious to try that combination was likewise irrelevant. Finally, the court held that genuine issues of material fact precluded summary judgment.

Held: The Federal Circuit addressed the obviousness question in a narrow, rigid manner that is inconsistent with §103 and this Court's

precedents. KSR provided convincing evidence that mounting an available sensor on a fixed pivot point of the Asano pedal was a design step well within the grasp of a person of ordinary skill in the relevant art and that the benefit of doing so would be obvious. Its arguments, and the record, demonstrate that the Engelgau patent's claim 4 is obvious. Pp. 11–24.

1. *Graham* provided an expansive and flexible approach to the obviousness question that is inconsistent with the way the Federal Circuit applied its TSM test here. Neither §103's enactment nor *Graham*'s analysis disturbed the Court's earlier instructions concerning the need for caution in granting a patent based on the combination of elements found in the prior art. See *Great Atlantic & Pacific Tea Co.* v. *Supermarket Equipment Corp.*, 340 U. S. 147, 152. Such a combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results. See, *e.g.*, *United States* v. *Adams*, 383 U. S. 39, 50–52. When a work is available in one field, design incentives and other market forces can prompt variations of it, either in the same field or in another. If a person of ordinary skill in the art can implement a predictable variation, and would see the benefit of doing so, §103 likely bars its patentability. Moreover, if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond that person's skill. A court must ask whether the improvement is more than the predictable use of prior-art elements according to their established functions. Following these principles may be difficult if the claimed subject matter involves more than the simple substitution of one known element for another or the mere application of a known technique to a piece of prior art ready for the improvement. To determine whether there was an apparent reason to combine the known elements in the way a patent claims, it will often be necessary to look to interrelated teachings of multiple patents; to the effects of demands known to the design community or present in the marketplace; and to the background knowledge possessed by a person having ordinary skill in the art. To facilitate review, this analysis should be made explicit. But it need not seek out precise teachings directed to the challenged claim's specific subject matter, for a court can consider the inferences and creative steps a person of ordinary skill in the art would employ. Pp. 11–14.

(b) The TSM test captures a helpful insight: A patent composed of several elements is not proved obvious merely by demonstrating that each element was, independently, known in the prior art. Although common sense directs caution as to a patent application claiming as

innovation the combination of two known devices according to their established functions, it can be important to identify a reason that would have prompted a person of ordinary skill in the art to combine the elements as the new invention does. Inventions usually rely upon building blocks long since uncovered, and claimed discoveries almost necessarily will be combinations of what, in some sense, is already known. Helpful insights, however, need not become rigid and mandatory formulas. If it is so applied, the TSM test is incompatible with this Court's precedents. The diversity of inventive pursuits and of modern technology counsels against confining the obviousness analysis by a formalistic conception of the words teaching, suggestion, and motivation, or by overemphasizing the importance of published articles and the explicit content of issued patents. In many fields there may be little discussion of obvious techniques or combinations, and market demand, rather than scientific literature, may often drive design trends. Granting patent protection to advances that would occur in the ordinary course without real innovation retards progress and may, for patents combining previously known elements, deprive prior inventions of their value or utility. Since the TSM test was devised, the Federal Circuit doubtless has applied it in accord with these principles in many cases. There is no necessary inconsistency between the test and the *Graham* analysis. But a court errs where, as here, it transforms general principle into a rigid rule limiting the obviousness inquiry. Pp. 14–15.

(c) The flaws in the Federal Circuit's analysis relate mostly to its narrow conception of the obviousness inquiry consequent in its application of the TSM test. The Circuit first erred in holding that courts and patent examiners should look only to the problem the patentee was trying to solve. Under the correct analysis, any need or problem known in the field and addressed by the patent can provide a reason for combining the elements in the manner claimed. Second, the appeals court erred in assuming that a person of ordinary skill in the art attempting to solve a problem will be led only to those prior art elements designed to solve the same problem. The court wrongly concluded that because Asano's primary purpose was solving the constant ratio problem, an inventor considering how to put a sensor on an adjustable pedal would have no reason to consider putting it on the Asano pedal. It is common sense that familiar items may have obvious uses beyond their primary purposes, and a person of ordinary skill often will be able to fit the teachings of multiple patents together like pieces of a puzzle. Regardless of Asano's primary purpose, it provided an obvious example of an adjustable pedal with a fixed pivot point, and the prior art was replete with patents indicating that such a point was an ideal mount for a sensor. Third, the

court erred in concluding that a patent claim cannot be proved obvious merely by showing that the combination of elements was obvious to try. When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill in the art has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense. Finally, the court drew the wrong conclusion from the risk of courts and patent examiners falling prey to hindsight bias. Rigid preventative rules that deny recourse to common sense are neither necessary under, nor consistent with, this Court's case law. Pp. 15–18.

2. Application of the foregoing standards demonstrates that claim 4 is obvious. Pp. 18–23.

(a) The Court rejects Teleflex's argument that the Asano pivot mechanism's design prevents its combination with a sensor in the manner claim 4 describes. This argument was not raised before the District Court, and it is unclear whether it was raised before the Federal Circuit. Given the significance of the District Court's finding that combining Asano with a pivot-mounted pedal position sensor fell within claim 4's scope, it is apparent that Teleflex would have made clearer challenges if it intended to preserve this claim. Its failure to clearly raise the argument, and the appeals court's silence on the issue, lead this Court to accept the District Court's conclusion. Pp. 18–20.

(b) The District Court correctly concluded that when Engelgau designed the claim 4 subject matter, it was obvious to a person of ordinary skill in the art to combine Asano with a pivot-mounted pedal position sensor. There then was a marketplace creating a strong incentive to convert mechanical pedals to electronic pedals, and the prior art taught a number of methods for doing so. The Federal Circuit considered the issue too narrowly by, in effect, asking whether a pedal designer writing on a blank slate would have chosen both Asano and a modular sensor similar to the ones used in the Chevrolet trucks and disclosed in the '068 patent. The proper question was whether a pedal designer of ordinary skill in the art, facing the wide range of needs created by developments in the field, would have seen an obvious benefit to upgrading Asano with a sensor. For such a designer starting with Asano, the question was where to attach the sensor. The '936 patent taught the utility of putting the sensor on the pedal device. Smith, in turn, explained not to put the sensor on the pedal footpad, but instead on the structure. And from Rixon's known wire-chafing problems, and Smith's teaching that the pedal assemblies must not precipitate any motion in the connecting wires,

the designer would know to place the sensor on a nonmoving part of the pedal structure. The most obvious such point is a pivot point. The designer, accordingly, would follow Smith in mounting the sensor there. Just as it was possible to begin with the objective to upgrade Asano to work with a computer-controlled throttle, so too was it possible to take an adjustable electronic pedal like Rixon and seek an improvement that would avoid the wire-chafing problem. Teleflex has not shown anything in the prior art that taught away from the use of Asano, nor any secondary factors to dislodge the determination that claim 4 is obvious. Pp. 20–23.

3. The Court disagrees with the Federal Circuit's holding that genuine issues of material fact precluded summary judgment. The ultimate judgment of obviousness is a legal determination. *Graham*, 383 U. S., at 17. Where, as here, the prior art's content, the patent claim's scope, and the level of ordinary skill in the art are not in material dispute and the claim's obviousness is apparent, summary judgment is appropriate. P. 23.

119 Fed. Appx. 282, reversed and remanded.

KENNEDY, J., delivered the opinion for a unanimous Court.

SUPREME COURT OF THE UNITED STATES

No. 04–1350

KSR INTERNATIONAL CO., PETITIONER *v.*
TELEFLEX INC. ET AL.

ON WRIT OF CERTIORARI TO THE UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

[April 30, 2007]

JUSTICE KENNEDY delivered the opinion of the Court.

Teleflex Incorporated and its subsidiary Technology Holding Company—both referred to here as Teleflex—sued KSR International Company for patent infringement. The patent at issue, United States Patent No. 6,237,565 B1, is entitled "Adjustable Pedal Assembly With Electronic Throttle Control." Supplemental App. 1. The patentee is Steven J. Engelgau, and the patent is referred to as "the Engelgau patent." Teleflex holds the exclusive license to the patent.

Claim 4 of the Engelgau patent describes a mechanism for combining an electronic sensor with an adjustable automobile pedal so the pedal's position can be transmitted to a computer that controls the throttle in the vehicle's engine. When Teleflex accused KSR of infringing the Engelgau patent by adding an electronic sensor to one of KSR's previously designed pedals, KSR countered that claim 4 was invalid under the Patent Act, 35 U. S. C. §103, because its subject matter was obvious.

Section 103 forbids issuance of a patent when "the differences between the subject matter sought to be pat-

ented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains."

In *Graham* v. *John Deere Co. of Kansas City*, 383 U. S. 1 (1966), the Court set out a framework for applying the statutory language of §103, language itself based on the logic of the earlier decision in *Hotchkiss* v. *Greenwood*, 11 How. 248 (1851), and its progeny. See 383 U. S., at 15–17. The analysis is objective:

> "Under §103, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented." *Id.*, at 17–18.

While the sequence of these questions might be reordered in any particular case, the factors continue to define the inquiry that controls. If a court, or patent examiner, conducts this analysis and concludes the claimed subject matter was obvious, the claim is invalid under §103.

Seeking to resolve the question of obviousness with more uniformity and consistency, the Court of Appeals for the Federal Circuit has employed an approach referred to by the parties as the "teaching, suggestion, or motivation" test (TSM test), under which a patent claim is only proved obvious if "some motivation or suggestion to combine the prior art teachings" can be found in the prior art, the nature of the problem, or the knowledge of a person having ordinary skill in the art. See, *e.g.*, *Al-Site Corp.* v. *VSI*

Int'l, Inc., 174 F. 3d 1308, 1323–1324 (CA Fed. 1999). KSR challenges that test, or at least its application in this case. See 119 Fed. Appx. 282, 286–290 (CA Fed. 2005). Because the Court of Appeals addressed the question of obviousness in a manner contrary to §103 and our precedents, we granted certiorari, 547 U. S ___ (2006). We now reverse.

I

A

In car engines without computer-controlled throttles, the accelerator pedal interacts with the throttle via cable or other mechanical link. The pedal arm acts as a lever rotating around a pivot point. In a cable-actuated throttle control the rotation caused by pushing down the pedal pulls a cable, which in turn pulls open valves in the carburetor or fuel injection unit. The wider the valves open, the more fuel and air are released, causing combustion to increase and the car to accelerate. When the driver takes his foot off the pedal, the opposite occurs as the cable is released and the valves slide closed.

In the 1990's it became more common to install computers in cars to control engine operation. Computer-controlled throttles open and close valves in response to electronic signals, not through force transferred from the pedal by a mechanical link. Constant, delicate adjustments of air and fuel mixture are possible. The computer's rapid processing of factors beyond the pedal's position improves fuel efficiency and engine performance.

For a computer-controlled throttle to respond to a driver's operation of the car, the computer must know what is happening with the pedal. A cable or mechanical link does not suffice for this purpose; at some point, an electronic sensor is necessary to translate the mechanical operation into digital data the computer can understand.

Before discussing sensors further we turn to the me-

chanical design of the pedal itself. In the traditional design a pedal can be pushed down or released but cannot have its position in the footwell adjusted by sliding the pedal forward or back. As a result, a driver who wishes to be closer or farther from the pedal must either reposition himself in the driver's seat or move the seat in some way. In cars with deep footwells these are imperfect solutions for drivers of smaller stature. To solve the problem, inventors, beginning in the 1970's, designed pedals that could be adjusted to change their location in the footwell. Important for this case are two adjustable pedals disclosed in U. S. Patent Nos. 5,010,782 (filed July 28, 1989) (Asano) and 5,460,061 (filed Sept. 17, 1993) (Redding). The Asano patent reveals a support structure that houses the pedal so that even when the pedal location is adjusted relative to the driver, one of the pedal's pivot points stays fixed. The pedal is also designed so that the force necessary to push the pedal down is the same regardless of adjustments to its location. The Redding patent reveals a different, sliding mechanism where both the pedal and the pivot point are adjusted.

We return to sensors. Well before Engelgau applied for his challenged patent, some inventors had obtained patents involving electronic pedal sensors for computer-controlled throttles. These inventions, such as the device disclosed in U. S. Patent No. 5,241,936 (filed Sept. 9, 1991) ('936), taught that it was preferable to detect the pedal's position in the pedal assembly, not in the engine. The '936 patent disclosed a pedal with an electronic sensor on a pivot point in the pedal assembly. U. S. Patent No. 5,063,811 (filed July 9, 1990) (Smith) taught that to prevent the wires connecting the sensor to the computer from chafing and wearing out, and to avoid grime and damage from the driver's foot, the sensor should be put on a fixed part of the pedal assembly rather than in or on the pedal's footpad.

In addition to patents for pedals with integrated sensors inventors obtained patents for self-contained modular sensors. A modular sensor is designed independently of a given pedal so that it can be taken off the shelf and attached to mechanical pedals of various sorts, enabling the pedals to be used in automobiles with computer-controlled throttles. One such sensor was disclosed in U. S. Patent No. 5,385,068 (filed Dec. 18, 1992) ('068). In 1994, Chevrolet manufactured a line of trucks using modular sensors "attached to the pedal support bracket, adjacent to the pedal and engaged with the pivot shaft about which the pedal rotates in operation." 298 F. Supp. 2d 581, 589 (ED Mich. 2003).

The prior art contained patents involving the placement of sensors on adjustable pedals as well. For example, U. S. Patent No. 5,819,593 (filed Aug. 17, 1995) (Rixon) discloses an adjustable pedal assembly with an electronic sensor for detecting the pedal's position. In the Rixon pedal the sensor is located in the pedal footpad. The Rixon pedal was known to suffer from wire chafing when the pedal was depressed and released.

This short account of pedal and sensor technology leads to the instant case.

B

KSR, a Canadian company, manufactures and supplies auto parts, including pedal systems. Ford Motor Company hired KSR in 1998 to supply an adjustable pedal system for various lines of automobiles with cable-actuated throttle controls. KSR developed an adjustable mechanical pedal for Ford and obtained U. S. Patent No. 6,151,976 (filed July 16, 1999) ('976) for the design. In 2000, KSR was chosen by General Motors Corporation (GMC or GM) to supply adjustable pedal systems for Chevrolet and GMC light trucks that used engines with computer-controlled throttles. To make the '976 pedal compatible with the

trucks, KSR merely took that design and added a modular sensor.

Teleflex is a rival to KSR in the design and manufacture of adjustable pedals. As noted, it is the exclusive licensee of the Engelgau patent. Engelgau filed the patent application on August 22, 2000 as a continuation of a previous application for U. S. Patent No. 6,109,241, which was filed on January 26, 1999. He has sworn he invented the patent's subject matter on February 14, 1998. The Engelgau patent discloses an adjustable electronic pedal described in the specification as a "simplified vehicle control pedal assembly that is less expensive, and which uses fewer parts and is easier to package within the vehicle." Engelgau, col. 2, lines 2–5, Supplemental App. 6. Claim 4 of the patent, at issue here, describes:

> "A vehicle control pedal apparatus comprising:
>
> a support adapted to be mounted to a vehicle structure;
>
> an adjustable pedal assembly having a pedal arm moveable in for[e] and aft directions with respect to said support;
>
> a pivot for pivotally supporting said adjustable pedal assembly with respect to said support and defining a pivot axis; and
>
> an electronic control attached to said support for controlling a vehicle system;
>
> said apparatus characterized by said electronic control being responsive to said pivot for providing a signal that corresponds to pedal arm position as said pedal arm pivots about said pivot axis between rest and applied positions wherein the position of said pivot remains constant while said pedal arm moves in fore and aft directions with respect to said pivot." *Id.*, col.

6, lines 17–36, Supplemental App. 8 (diagram numbers omitted).

We agree with the District Court that the claim discloses "a position-adjustable pedal assembly with an electronic pedal position sensor attached to the support member of the pedal assembly. Attaching the sensor to the support member allows the sensor to remain in a fixed position while the driver adjusts the pedal." 298 F. Supp. 2d, at 586–587.

Before issuing the Engelgau patent the U. S. Patent and Trademark Office (PTO) rejected one of the patent claims that was similar to, but broader than, the present claim 4. The claim did not include the requirement that the sensor be placed on a fixed pivot point. The PTO concluded the claim was an obvious combination of the prior art disclosed in Redding and Smith, explaining:

> "'Since the prior ar[t] references are from the field of endeavor, the purpose disclosed . . . would have been recognized in the pertinent art of Redding. Therefore it would have been obvious . . . to provide the device of Redding with the . . . means attached to a support member as taught by Smith.'" *Id.*, at 595.

In other words Redding provided an example of an adjustable pedal and Smith explained how to mount a sensor on a pedal's support structure, and the rejected patent claim merely put these two teachings together.

Although the broader claim was rejected, claim 4 was later allowed because it included the limitation of a fixed pivot point, which distinguished the design from Redding's. *Ibid.* Engelgau had not included Asano among the prior art references, and Asano was not mentioned in the patent's prosecution. Thus, the PTO did not have before it an adjustable pedal with a fixed pivot point. The patent issued on May 29, 2001 and was assigned to Teleflex.

Upon learning of KSR's design for GM, Teleflex sent a

warning letter informing KSR that its proposal would violate the Engelgau patent. "'Teleflex believes that any supplier of a product that combines an adjustable pedal with an electronic throttle control necessarily employs technology covered by one or more'" of Teleflex's patents. *Id.*, at 585. KSR refused to enter a royalty arrangement with Teleflex; so Teleflex sued for infringement, asserting KSR's pedal infringed the Engelgau patent and two other patents. *Ibid.* Teleflex later abandoned its claims regarding the other patents and dedicated the patents to the public. The remaining contention was that KSR's pedal system for GM infringed claim 4 of the Engelgau patent. Teleflex has not argued that the other three claims of the patent are infringed by KSR's pedal, nor has Teleflex argued that the mechanical adjustable pedal designed by KSR for Ford infringed any of its patents.

C

The District Court granted summary judgment in KSR's favor. After reviewing the pertinent history of pedal design, the scope of the Engelgau patent, and the relevant prior art, the court considered the validity of the contested claim. By direction of 35 U. S. C. §282, an issued patent is presumed valid. The District Court applied *Graham*'s framework to determine whether under summary-judgment standards KSR had overcome the presumption and demonstrated that claim 4 was obvious in light of the prior art in existence when the claimed subject matter was invented. See §102(a).

The District Court determined, in light of the expert testimony and the parties' stipulations, that the level of ordinary skill in pedal design was "'an undergraduate degree in mechanical engineering (or an equivalent amount of industry experience) [and] familiarity with pedal control systems for vehicles.'" 298 F. Supp. 2d, at 590. The court then set forth the relevant prior art, in-

cluding the patents and pedal designs described above.

Following *Graham*'s direction, the court compared the teachings of the prior art to the claims of Engelgau. It found "little difference." 298 F. Supp. 2d, at 590. Asano taught everything contained in claim 4 except the use of a sensor to detect the pedal's position and transmit it to the computer controlling the throttle. That additional aspect was revealed in sources such as the '068 patent and the sensors used by Chevrolet.

Under the controlling cases from the Court of Appeals for the Federal Circuit, however, the District Court was not permitted to stop there. The court was required also to apply the TSM test. The District Court held KSR had satisfied the test. It reasoned (1) the state of the industry would lead inevitably to combinations of electronic sensors and adjustable pedals, (2) Rixon provided the basis for these developments, and (3) Smith taught a solution to the wire chafing problems in Rixon, namely locating the sensor on the fixed structure of the pedal. This could lead to the combination of Asano, or a pedal like it, with a pedal position sensor.

The conclusion that the Engelgau design was obvious was supported, in the District Court's view, by the PTO's rejection of the broader version of claim 4. Had Engelgau included Asano in his patent application, it reasoned, the PTO would have found claim 4 to be an obvious combination of Asano and Smith, as it had found the broader version an obvious combination of Redding and Smith. As a final matter, the District Court held that the secondary factor of Teleflex's commercial success with pedals based on Engelgau's design did not alter its conclusion. The District Court granted summary judgment for KSR.

With principal reliance on the TSM test, the Court of Appeals reversed. It ruled the District Court had not been strict enough in applying the test, having failed to make "'finding[s] as to the specific understanding or principle

within the knowledge of a skilled artisan that would have motivated one with no knowledge of [the] invention' . . . to attach an electronic control to the support bracket of the Asano assembly." 119 Fed. Appx., at 288 (brackets in original) (quoting *In re Kotzab*, 217 F. 3d 1365, 1371 (CA Fed. 2000)). The Court of Appeals held that the District Court was incorrect that the nature of the problem to be solved satisfied this requirement because unless the "prior art references address[ed] the precise problem that the patentee was trying to solve," the problem would not motivate an inventor to look at those references. 119 Fed. Appx., at 288.

Here, the Court of Appeals found, the Asano pedal was designed to solve the "'constant ratio problem'"—that is, to ensure that the force required to depress the pedal is the same no matter how the pedal is adjusted—whereas Engelgau sought to provide a simpler, smaller, cheaper adjustable electronic pedal. *Ibid.* As for Rixon, the court explained, that pedal suffered from the problem of wire chafing but was not designed to solve it. In the court's view Rixon did not teach anything helpful to Engelgau's purpose. Smith, in turn, did not relate to adjustable pedals and did not "necessarily go to the issue of motivation to attach the electronic control on the support bracket of the pedal assembly." *Ibid.* When the patents were interpreted in this way, the Court of Appeals held, they would not have led a person of ordinary skill to put a sensor on the sort of pedal described in Asano.

That it might have been obvious to try the combination of Asano and a sensor was likewise irrelevant, in the court's view, because "'"[o]bvious to try" has long been held not to constitute obviousness.'" *Id.*, at 289 (quoting *In re Deuel*, 51 F. 3d 1552, 1559 (CA Fed. 1995)).

The Court of Appeals also faulted the District Court's consideration of the PTO's rejection of the broader version of claim 4. The District Court's role, the Court of Appeals

explained, was not to speculate regarding what the PTO might have done had the Engelgau patent mentioned Asano. Rather, the court held, the District Court was obliged first to presume that the issued patent was valid and then to render its own independent judgment of obviousness based on a review of the prior art. The fact that the PTO had rejected the broader version of claim 4, the Court of Appeals said, had no place in that analysis.

The Court of Appeals further held that genuine issues of material fact precluded summary judgment. Teleflex had proffered statements from one expert that claim 4 "'was a simple, elegant, and novel combination of features,'" 119 Fed. Appx., at 290, compared to Rixon, and from another expert that claim 4 was nonobvious because, unlike in Rixon, the sensor was mounted on the support bracket rather than the pedal itself. This evidence, the court concluded, sufficed to require a trial.

II

A

We begin by rejecting the rigid approach of the Court of Appeals. Throughout this Court's engagement with the question of obviousness, our cases have set forth an expansive and flexible approach inconsistent with the way the Court of Appeals applied its TSM test here. To be sure, *Graham* recognized the need for "uniformity and definiteness." 383 U. S., at 18. Yet the principles laid down in *Graham* reaffirmed the "functional approach" of *Hotchkiss*, 11 How. 248. See 383 U. S., at 12. To this end, *Graham* set forth a broad inquiry and invited courts, where appropriate, to look at any secondary considerations that would prove instructive. *Id.*, at 17.

Neither the enactment of §103 nor the analysis in *Graham* disturbed this Court's earlier instructions concerning the need for caution in granting a patent based on the combination of elements found in the prior art. For over a

half century, the Court has held that a "patent for a combination which only unites old elements with no change in their respective functions . . . obviously withdraws what is already known into the field of its monopoly and diminishes the resources available to skillful men." *Great Atlantic & Pacific Tea Co.* v. *Supermarket Equipment Corp.*, 340 U. S. 147, 152 (1950). This is a principal reason for declining to allow patents for what is obvious. The combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results. Three cases decided after *Graham* illustrate the application of this doctrine.

In *United States* v. *Adams*, 383 U. S. 39, 40 (1966), a companion case to *Graham*, the Court considered the obviousness of a "wet battery" that varied from prior designs in two ways: It contained water, rather than the acids conventionally employed in storage batteries; and its electrodes were magnesium and cuprous chloride, rather than zinc and silver chloride. The Court recognized that when a patent claims a structure already known in the prior art that is altered by the mere substitution of one element for another known in the field, the combination must do more than yield a predictable result. 383 U. S., at 50–51. It nevertheless rejected the Government's claim that Adams's battery was obvious. The Court relied upon the corollary principle that when the prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be nonobvious. *Id.*, at 51–52. When Adams designed his battery, the prior art warned that risks were involved in using the types of electrodes he employed. The fact that the elements worked together in an unexpected and fruitful manner supported the conclusion that Adams's design was not obvious to those skilled in the art.

In *Anderson's-Black Rock, Inc.* v. *Pavement Salvage Co.*, 396 U. S. 57 (1969), the Court elaborated on this approach.

The subject matter of the patent before the Court was a device combining two pre-existing elements: a radiant-heat burner and a paving machine. The device, the Court concluded, did not create some new synergy: The radiant-heat burner functioned just as a burner was expected to function; and the paving machine did the same. The two in combination did no more than they would in separate, sequential operation. *Id.*, at 60–62. In those circumstances, "while the combination of old elements performed a useful function, it added nothing to the nature and quality of the radiant-heat burner already patented," and the patent failed under §103. *Id.*, at 62 (footnote omitted).

Finally, in *Sakraida* v. *AG Pro, Inc.*, 425 U. S. 273 (1976), the Court derived from the precedents the conclusion that when a patent "simply arranges old elements with each performing the same function it had been known to perform" and yields no more than one would expect from such an arrangement, the combination is obvious. *Id.*, at 282.

The principles underlying these cases are instructive when the question is whether a patent claiming the combination of elements of prior art is obvious. When a work is available in one field of endeavor, design incentives and other market forces can prompt variations of it, either in the same field or a different one. If a person of ordinary skill can implement a predictable variation, §103 likely bars its patentability. For the same reason, if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill. *Sakraida* and *Anderson's-Black Rock* are illustrative—a court must ask whether the improvement is more than the predictable use of prior art elements according to their established functions.

Following these principles may be more difficult in other

cases than it is here because the claimed subject matter may involve more than the simple substitution of one known element for another or the mere application of a known technique to a piece of prior art ready for the improvement. Often, it will be necessary for a court to look to interrelated teachings of multiple patents; the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person having ordinary skill in the art, all in order to determine whether there was an apparent reason to combine the known elements in the fashion claimed by the patent at issue. To facilitate review, this analysis should be made explicit. See *In re Kahn*, 441 F. 3d 977, 988 (CA Fed. 2006) ("[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness"). As our precedents make clear, however, the analysis need not seek out precise teachings directed to the specific subject matter of the challenged claim, for a court can take account of the inferences and creative steps that a person of ordinary skill in the art would employ.

B

When it first established the requirement of demonstrating a teaching, suggestion, or motivation to combine known elements in order to show that the combination is obvious, the Court of Customs and Patent Appeals captured a helpful insight. See *Application of Bergel*, 292 F. 2d 955, 956–957 (1961). As is clear from cases such as *Adams*, a patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art. Although common sense directs one to look with care at a patent application that claims as innovation the combination of two known devices according to their established

functions, it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does. This is so because inventions in most, if not all, instances rely upon building blocks long since uncovered, and claimed discoveries almost of necessity will be combinations of what, in some sense, is already known.

Helpful insights, however, need not become rigid and mandatory formulas; and when it is so applied, the TSM test is incompatible with our precedents. The obviousness analysis cannot be confined by a formalistic conception of the words teaching, suggestion, and motivation, or by overemphasis on the importance of published articles and the explicit content of issued patents. The diversity of inventive pursuits and of modern technology counsels against limiting the analysis in this way. In many fields it may be that there is little discussion of obvious techniques or combinations, and it often may be the case that market demand, rather than scientific literature, will drive design trends. Granting patent protection to advances that would occur in the ordinary course without real innovation retards progress and may, in the case of patents combining previously known elements, deprive prior inventions of their value or utility.

In the years since the Court of Customs and Patent Appeals set forth the essence of the TSM test, the Court of Appeals no doubt has applied the test in accord with these principles in many cases. There is no necessary inconsistency between the idea underlying the TSM test and the *Graham* analysis. But when a court transforms the general principle into a rigid rule that limits the obviousness inquiry, as the Court of Appeals did here, it errs.

C

The flaws in the analysis of the Court of Appeals relate

for the most part to the court's narrow conception of the obviousness inquiry reflected in its application of the TSM test. In determining whether the subject matter of a patent claim is obvious, neither the particular motivation nor the avowed purpose of the patentee controls. What matters is the objective reach of the claim. If the claim extends to what is obvious, it is invalid under §103. One of the ways in which a patent's subject matter can be proved obvious is by noting that there existed at the time of invention a known problem for which there was an obvious solution encompassed by the patent's claims.

The first error of the Court of Appeals in this case was to foreclose this reasoning by holding that courts and patent examiners should look only to the problem the patentee was trying to solve. 119 Fed. Appx., at 288. The Court of Appeals failed to recognize that the problem motivating the patentee may be only one of many addressed by the patent's subject matter. The question is not whether the combination was obvious to the patentee but whether the combination was obvious to a person with ordinary skill in the art. Under the correct analysis, any need or problem known in the field of endeavor at the time of invention and addressed by the patent can provide a reason for combining the elements in the manner claimed.

The second error of the Court of Appeals lay in its assumption that a person of ordinary skill attempting to solve a problem will be led only to those elements of prior art designed to solve the same problem. *Ibid.* The primary purpose of Asano was solving the constant ratio problem; so, the court concluded, an inventor considering how to put a sensor on an adjustable pedal would have no reason to consider putting it on the Asano pedal. *Ibid.* Common sense teaches, however, that familiar items may have obvious uses beyond their primary purposes, and in many cases a person of ordinary skill will be able to fit the teachings of multiple patents together like pieces of a

puzzle. Regardless of Asano's primary purpose, the design provided an obvious example of an adjustable pedal with a fixed pivot point; and the prior art was replete with patents indicating that a fixed pivot point was an ideal mount for a sensor. The idea that a designer hoping to make an adjustable electronic pedal would ignore Asano because Asano was designed to solve the constant ratio problem makes little sense. A person of ordinary skill is also a person of ordinary creativity, not an automaton.

The same constricted analysis led the Court of Appeals to conclude, in error, that a patent claim cannot be proved obvious merely by showing that the combination of elements was "obvious to try." *Id.*, at 289 (internal quotation marks omitted). When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense. In that instance the fact that a combination was obvious to try might show that it was obvious under §103.

The Court of Appeals, finally, drew the wrong conclusion from the risk of courts and patent examiners falling prey to hindsight bias. A factfinder should be aware, of course, of the distortion caused by hindsight bias and must be cautious of arguments reliant upon *ex post* reasoning. See *Graham*, 383 U. S., at 36 (warning against a "temptation to read into the prior art the teachings of the invention in issue" and instructing courts to "'guard against slipping into the use of hindsight'" (quoting *Monroe Auto Equipment Co.* v. *Heckethorn Mfg. & Supply Co.*, 332 F. 2d 406, 412 (CA6 1964))). Rigid preventative rules that deny factfinders recourse to common sense, however, are neither necessary under our case law nor consistent with it.

We note the Court of Appeals has since elaborated a

broader conception of the TSM test than was applied in the instant matter. See, *e.g., DyStar Textilfarben GmbH & Co. Deutschland KG* v. *C. H. Patrick Co.*, 464 F. 3d 1356, 1367 (2006) ("Our suggestion test is in actuality quite flexible and not only permits, but *requires*, consideration of common knowledge and common sense"); *Alza Corp.* v. *Mylan Labs., Inc.*, 464 F. 3d 1286, 1291 (2006) ("There is flexibility in our obviousness jurisprudence because a motivation may be found *implicitly* in the prior art. We do not have a rigid test that requires an actual teaching to combine . . ."). Those decisions, of course, are not now before us and do not correct the errors of law made by the Court of Appeals in this case. The extent to which they may describe an analysis more consistent with our earlier precedents and our decision here is a matter for the Court of Appeals to consider in its future cases. What we hold is that the fundamental misunderstandings identified above led the Court of Appeals in this case to apply a test inconsistent with our patent law decisions.

III

When we apply the standards we have explained to the instant facts, claim 4 must be found obvious. We agree with and adopt the District Court's recitation of the relevant prior art and its determination of the level of ordinary skill in the field. As did the District Court, we see little difference between the teachings of Asano and Smith and the adjustable electronic pedal disclosed in claim 4 of the Engelgau patent. A person having ordinary skill in the art could have combined Asano with a pedal position sensor in a fashion encompassed by claim 4, and would have seen the benefits of doing so.

A

Teleflex argues in passing that the Asano pedal cannot be combined with a sensor in the manner described by

claim 4 because of the design of Asano's pivot mechanisms. See Brief for Respondents 48–49, and n. 17. Therefore, Teleflex reasons, even if adding a sensor to Asano was obvious, that does not establish that claim 4 encompasses obvious subject matter. This argument was not, however, raised before the District Court. There Teleflex was content to assert only that the problem motivating the invention claimed by the Engelgau patent would not lead to the solution of combining of Asano with a sensor. See Teleflex's Response to KSR's Motion for Summary Judgment of Invalidity in No. 02–74586 (ED Mich.), pp. 18–20, App. 144a–146a. It is also unclear whether the current argument was raised before the Court of Appeals, where Teleflex advanced the nonspecific, conclusory contention that combining Asano with a sensor would not satisfy the limitations of claim 4. See Brief for Plaintiffs-Appellants in No. 04–1152 (CA Fed.), pp. 42–44. Teleflex's own expert declarations, moreover, do not support the point Teleflex now raises. See Declaration of Clark J. Radcliffe, Ph.D., Supplemental App. 204–207; Declaration of Timothy L. Andresen, *id.*, at 208–210. The only statement in either declaration that might bear on the argument is found in the Radcliffe declaration:

> "Asano . . . and Rixon . . . are complex mechanical linkage-based devices that are expensive to produce and assemble and difficult to package. It is exactly these difficulties with prior art designs that [Engelgau] resolves. The use of an adjustable pedal with a single pivot reflecting pedal position combined with an electronic control mounted between the support and the adjustment assembly at that pivot was a simple, elegant, and novel combination of features in the Engelgau '565 patent." *Id.*, at 206, ¶16.

Read in the context of the declaration as a whole this is best interpreted to mean that Asano could not be used to

solve "[t]he problem addressed by Engelgau '565[:] to provide a less expensive, more quickly assembled, and smaller package adjustable pedal assembly with electronic control." *Id.*, at 205, ¶10.

The District Court found that combining Asano with a pivot-mounted pedal position sensor fell within the scope of claim 4. 298 F. Supp. 2d, at 592–593. Given the sigificance of that finding to the District Court's judgment, it is apparent that Teleflex would have made clearer challenges to it if it intended to preserve this claim. In light of Teleflex's failure to raise the argument in a clear fashion, and the silence of the Court of Appeals on the issue, we take the District Court's conclusion on the point to be correct.

B

The District Court was correct to conclude that, as of the time Engelgau designed the subject matter in claim 4, it was obvious to a person of ordinary skill to combine Asano with a pivot-mounted pedal position sensor. There then existed a marketplace that created a strong incentive to convert mechanical pedals to electronic pedals, and the prior art taught a number of methods for achieving this advance. The Court of Appeals considered the issue too narrowly by, in effect, asking whether a pedal designer writing on a blank slate would have chosen both Asano and a modular sensor similar to the ones used in the Chevrolet truckline and disclosed in the '068 patent. The District Court employed this narrow inquiry as well, though it reached the correct result nevertheless. The proper question to have asked was whether a pedal designer of ordinary skill, facing the wide range of needs created by developments in the field of endeavor, would have seen a benefit to upgrading Asano with a sensor.

In automotive design, as in many other fields, the interaction of multiple components means that changing one

component often requires the others to be modified as well. Technological developments made it clear that engines using computer-controlled throttles would become standard. As a result, designers might have decided to design new pedals from scratch; but they also would have had reason to make pre-existing pedals work with the new engines. Indeed, upgrading its own pre-existing model led KSR to design the pedal now accused of infringing the Engelgau patent.

For a designer starting with Asano, the question was where to attach the sensor. The consequent legal question, then, is whether a pedal designer of ordinary skill starting with Asano would have found it obvious to put the sensor on a fixed pivot point. The prior art discussed above leads us to the conclusion that attaching the sensor where both KSR and Engelgau put it would have been obvious to a person of ordinary skill.

The '936 patent taught the utility of putting the sensor on the pedal device, not in the engine. Smith, in turn, explained to put the sensor not on the pedal's footpad but instead on its support structure. And from the known wire-chafing problems of Rixon, and Smith's teaching that "the pedal assemblies must not precipitate any motion in the connecting wires," Smith, col. 1, lines 35–37, Supplemental App. 274, the designer would know to place the sensor on a nonmoving part of the pedal structure. The most obvious nonmoving point on the structure from which a sensor can easily detect the pedal's position is a pivot point. The designer, accordingly, would follow Smith in mounting the sensor on a pivot, thereby designing an adjustable electronic pedal covered by claim 4.

Just as it was possible to begin with the objective to upgrade Asano to work with a computer-controlled throttle, so too was it possible to take an adjustable electronic pedal like Rixon and seek an improvement that would avoid the wire-chafing problem. Following similar steps to

those just explained, a designer would learn from Smith to avoid sensor movement and would come, thereby, to Asano because Asano disclosed an adjustable pedal with a fixed pivot.

Teleflex indirectly argues that the prior art taught away from attaching a sensor to Asano because Asano in its view is bulky, complex, and expensive. The only evidence Teleflex marshals in support of this argument, however, is the Radcliffe declaration, which merely indicates that Asano would not have solved Engelgau's goal of making a small, simple, and inexpensive pedal. What the declaration does not indicate is that Asano was somehow so flawed that there was no reason to upgrade it, or pedals like it, to be compatible with modern engines. Indeed, Teleflex's own declarations refute this conclusion. Dr. Radcliffe states that Rixon suffered from the same bulk and complexity as did Asano. See *id.*, at 206. Teleflex's other expert, however, explained that Rixon was itself designed by adding a sensor to a pre-existing mechanical pedal. See *id.*, at 209. If Rixon's base pedal was not too flawed to upgrade, then Dr. Radcliffe's declaration does not show Asano was either. Teleflex may have made a plausible argument that Asano is inefficient as compared to Engelgau's preferred embodiment, but to judge Asano against Engelgau would be to engage in the very hindsight bias Teleflex rightly urges must be avoided. Accordingly, Teleflex has not shown anything in the prior art that taught away from the use of Asano.

Like the District Court, finally, we conclude Teleflex has shown no secondary factors to dislodge the determination that claim 4 is obvious. Proper application of *Graham* and our other precedents to these facts therefore leads to the conclusion that claim 4 encompassed obvious subject matter. As a result, the claim fails to meet the requirement of §103.

We need not reach the question whether the failure to

disclose Asano during the prosecution of Engelgau voids the presumption of validity given to issued patents, for claim 4 is obvious despite the presumption. We nevertheless think it appropriate to note that the rationale underlying the presumption—that the PTO, in its expertise, has approved the claim—seems much diminished here.

IV

A separate ground the Court of Appeals gave for reversing the order for summary judgment was the existence of a dispute over an issue of material fact. We disagree with the Court of Appeals on this point as well. To the extent the court understood the *Graham* approach to exclude the possibility of summary judgment when an expert provides a conclusory affidavit addressing the question of obviousness, it misunderstood the role expert testimony plays in the analysis. In considering summary judgment on that question the district court can and should take into account expert testimony, which may resolve or keep open certain questions of fact. That is not the end of the issue, however. The ultimate judgment of obviousness is a legal determination. *Graham*, 383 U. S., at 17. Where, as here, the content of the prior art, the scope of the patent claim, and the level of ordinary skill in the art are not in material dispute, and the obviousness of the claim is apparent in light of these factors, summary judgment is appropriate. Nothing in the declarations proffered by Teleflex prevented the District Court from reaching the careful conclusions underlying its order for summary judgment in this case.

* * *

We build and create by bringing to the tangible and palpable reality around us new works based on instinct, simple logic, ordinary inferences, extraordinary ideas, and sometimes even genius. These advances, once part of our

shared knowledge, define a new threshold from which innovation starts once more. And as progress beginning from higher levels of achievement is expected in the normal course, the results of ordinary innovation are not the subject of exclusive rights under the patent laws. Were it otherwise patents might stifle, rather than promote, the progress of useful arts. See U. S. Const., Art. I, §8, cl. 8. These premises led to the bar on patents claiming obvious subject matter established in *Hotchkiss* and codified in §103. Application of the bar must not be confined within a test or formulation too constrained to serve its purpose.

KSR provided convincing evidence that mounting a modular sensor on a fixed pivot point of the Asano pedal was a design step well within the grasp of a person of ordinary skill in the relevant art. Its arguments, and the record, demonstrate that claim 4 of the Engelgau patent is obvious. In rejecting the District Court's rulings, the Court of Appeals analyzed the issue in a narrow, rigid manner inconsistent with §103 and our precedents. The judgment of the Court of Appeals is reversed, and the case remanded for further proceedings consistent with this opinion.

It is so ordered.

IN RE CHARLES v. HEDGES and VICTOR MARK

No. 85-2524

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

783 F.2d 1038; 1986 U.S. App. LEXIS 20001; 228 U.S.P.Q. (BNA) 685

February 12, 1986

PRIOR HISTORY: [**1] Appealed from: U.S. Patent and Trademark Office Board of Patent Appeals and Interferences.

CASE SUMMARY:

PROCEDURAL POSTURE: Patent applicants appealed from a decision of the U.S. Patent and Trademark Office Board of Patent Appeals and Interferences Board of Appeals, affirming the rejection of applicants' claims for a process as unpatentable under 35 U.S.C.S. § 103.

OVERVIEW: Appellant patent applicants challenged the rejection of appellants' claims for a process as unpatentable under 35 U.S.C.S. § 103. The patent board had held that a particular prior reference that mentioned the lower temperature that was a part of appellants' process, without more, made a prima facie case of obviousness. The court on appeal did not agree, and found that other cited references supported appellants' position. Appellants argued that the earlier belief held by those skilled in the art was that lower temperatures were needed in the type of reaction at issue, which view was supported with declarations of record. The court held that the prior art must be considered as a whole, and was to be viewed as it would have been viewed by one of ordinary skill. The court found that appellants had proceeded contrary to the accepted wisdom and that was strong evidence of non-obviousness.

OUTCOME: The decision affirming the rejection of applicants' claims for a process as unpatentable was reversed because the prior art as a whole supported a finding of non-obviousness.

LexisNexis(R) Headnotes

Evidence > Inferences & Presumptions > General Overview
Patent Law > Nonobviousness > Elements & Tests > General Overview
Patent Law > Nonobviousness > Evidence & Procedure > Prima Facie Obviousness
[HN1]Only after the United States Patent and Trademark Office has made a prima facie case of obviousness does the burden of coming forward shift to the applicant.

Evidence > Inferences & Presumptions > General Overview
Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN2]If a prima facie case is made in the first instance in a case of obviousness and if the applicant comes forward with reasonable rebuttal, whether buttressed by experiment, prior art references, or argument, the entire merits of the matter are to be reevaluated.

Civil Procedure > Appeals > Reviewability > Preservation for Review
Patent Law > Jurisdiction & Review > General Overview
[HN3]The United States Court of Appeals for the Federal Circuit does not condone the presentation of new grounds of rejection for the first time on appeal.

Patent Law > Anticipation & Novelty > General Overview
Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN4]The prior art as a whole must be considered. The teachings are to be viewed as they would have been viewed by one of ordinary skill.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN5]Where one proceeds contrary to the accepted wisdom it is "strong evidence of nonobviousness."

COUNSEL: Martin B. Barancik, of Mount Vernon, Indiana, argued for Appellants. With him on the brief was John W. Schneller, Lyon & Lyon, of Washington, District of Columbia.

Henry W. Tarring, Associate Solicitor, Office of the Solicitor, of Arlington, Virginia, argues for Appellee. With him on the brief were Joseph F. Nakamura, Solicitor and Fred E. McKelvey, Deputy Solicitor.

JUDGES: Markey, Chief Judge, Miller, Senior Circuit Judge, and Newman, Circuit Judge.

OPINION BY: NEWMAN

OPINION

[*1038] NEWMAN, Circuit Judge.

The decision of the United States Patent and Trademark Office (PTO) Board of Appeals (Board), affirming the rejection of claims 8, 9, and 10 of United States patent application Serial No. 301,396 as unpatentable under 35 U.S.C. § 103, is reversed.

OPINION

This patent application of Charles V. Hedges and Victor Mark (collectively Hedges or applicant) is for a "Process for Preparing [*1039] Aryl Sulfone Sulfonic Acids". Claim 8 is representative:

> 8. A process for sulfonating diphenyl sulfone which comprises contacting diphenyl [**2] sulfone in its molten state with a sulfonating agent consisting essentially of sulfur trioxide under substantially anhydrous conditions in the absence of a solvent.

Hedges' invention is the reaction of diphenyl sulfone, at a temperature above its melting point of 127 degrees C, with liquid or gaseous sulfur trioxide in the absence of water or a solvent, thereby sulfonating the sulfone in high yields without forming by-product sulfuric acid.

The only rejection is under 35 U.S.C. § 103, and the Board relied only on Felix U.S. Patent No. 2,010,754. Hedges has cited three additional references, parts of which were discussed by the Board: Mark U.S. Patent No. 3,948,851, British Patent No. 820,659, and certain pages of a book by Gilbert entitled "Sulfonation and Related Reactions". The PTO Solicitor on this appeal discusses and relies on all these references.

Felix shows the sulfonation of aryl sulfones with sulfur trioxide in the form of fuming sulphuric acid. Sulfonation is carried out at 5-10 degrees C, after which the temperature rises exothermically to 30 degrees C before it is lowered to room temperature. The Board held that this, without [**3] more, makes a prima facie case of obviousness.

Hedges has taken the position, before the Board and before us, that the low temperatures shown by Felix defeat any prima facie case of obviousness of the reaction at above 127 degrees C. Hedges also argues that, viewing the references as a whole, it would not have been obvious to operate in the molten state at high temperatures. The Board held that Hedges had not produced "persuasive objective evidence" in rebuttal.

[HN1]Only after the PTO has made a prima facie case of obviousness does the burden of coming forward shift to the applicant. *In re Rinehart*, 531 F.2d 1048, 1051, 189 U.S.P.Q. (BNA) 143, 147 (CCPA 1976). [HN2]If a prima facie case is made in the first instance, and if the applicant comes forward with reasonable rebuttal, whether buttressed by experiment, prior art references, or argument, the entire merits of the matter

are to be reweighed. *In re Piasecki*, 745 F.2d 1468, 1472, 223 U.S.P.Q. (BNA) 785, 788 (Fed. Cir. 1984). [**4]

In the case before us, we do not agree with the PTO that Felix alone supports a prima facie case of obviousness. Felix makes clear that low temperatures are the desired conditions for this reaction. However, the Solicitor has elaborated on and strengthened the PTO argument by drawing on the additional prior art cited by Hedges. Hedges takes vigorous exception to this procedure, arguing that he has been deprived of the opportunity to respond before the PTO to these "new grounds of rejection" and to produce evidence in rebuttal.

[HN3]We and our predecessor court have not condoned the presentation of new grounds of rejection for the first time on appeal. *In re Hounsfield*, 699 F.2d 1320, 1324, 216 U.S.P.Q. (BNA) 1045, 1049 (Fed. Cir. 1982); *In re Zeidler*, 682 F.2d 961, 967, 215 U.S.P.Q. (BNA) 490, 494 (CCPA 1982); *In re Nygard*, 52 C.C.P.A. 1032, 341 F.2d 924, 928-9, 144 U.S.P.Q. (BNA) 586, 590 (CCPA 1965). In Hedges' case the Solicitor referred to new portions of the references cited by Hedges during examination for further support of the [**5] same rejection that had been upheld by the Board. Hedges had relied on these references before the Board, as he does before us, for his argument that viewed as a whole the body of prior art teaches away from conducting this reaction at high temperatures. The Solicitor should not be constrained from pointing to other portions of these same references in contravention of Hedges' position. *In re Wesslau*, 53 C.C.P.A. 746, 353 F.2d 238, 241, 147 U.S.P.Q. (BNA) 391, 393 (CCPA 1965) (the reference is considered in its entirety for what it fairly suggests to one skilled in the art). On these facts, we do not discern that the Solicitor has violated the rule against presenting new issues on appeal. The Solicitor has done no more than search the references of record for disclosures pertinent [*1040] to the same arguments for which Hedges cited the references.

The PTO argues that Felix shows no upper limit to the temperature of the reaction, and that determining the optimum temperature is a matter of "routine experimentation". The plain reading of Felix is contrary to the PTO position. As was said in *In re Rosenberger*, 55 C.C.P.A. 880, 386 F.2d 1015, 1018, 156 U.S.P.Q. (BNA) 24, 26 (CCPA 1967), [**6] "this appears to be an extremely strained interpretation of the reference which could be made only by hindsight."

To overcome this deficiency in Felix the Solicitor directs attention to the British patent, which discusses the reaction of liquid phenols with liquid sulfur trioxide in the absence of a solvent. The PTO points to the teachings of reaction at elevated temperature:

> The invention is applicable to liquid and solid phenols . . . having melting points up to 115 degrees C . . . and to mixtures of phenols whose individual melting point is higher than 115 degrees C but which give in admixture a melting point of 115 degrees C or lower.
>
> For mono-sulphonic acids . . . the temperature is kept above the melting point of the phenol used.
>
> . . . the liquid sulphur trioxide is added . . . at a temperature slightly above the melting point of the phenol in the case of solid phenols, and after the addition the reaction mass is heated at a higher temperature of 160-180 degrees C. . . .

The highest-melting phenol illustrated in the British patent is resorcinol, melting point 110 degrees C, to which

> liquid sulfur trioxide is added . . . at a temperature of 115-140 degrees [**7] C. . . . The product, which is almost black in colour and sets to a brittle solid on cooling, is substantially the monosulphonic acid in quantitative yield.

The Solicitor asserts that this shows that aromatic compounds can be sulfonated, in the absence of solvent, in the molten state, at the temperatures contemplated by Hedges. Hedges argues that the British patent expressly teaches that the reaction cannot be carried out with phenols that melt higher than 115 degrees, that the upper temperature range reported for resorcinol is reached during the exothermic reaction, and that the black color and brittle product are due to charring and decomposition. Hedges argues that the British patent does not negate the overall teachings of the art as a whole that lower temperatures are preferred for optimum results, and that the charring at higher temperatures that is shown in the British patent belies the broad conclusion that the Solicitor attempts to draw. The cited references support

Hedges' position.

The Mark patent shows diphenyl sulfone sulfonated with sulfur trioxide and states that by "well known methods . . . these reactions can be carried out at room temperature or at [**8] elevated temperatures such as about 50 degrees C". Mark, who is co-inventor herein, has averred that reaction at 50 degrees C obviously requires the presence of a solvent, because diphenyl sulfone is a solid at 50 degrees C. The PTO does not dispute this point. We do not agree with the Solicitor that Mark is an open-ended teaching of the use of higher temperatures, such as over 127 degrees C, for this reaction, merely because Mark does not state that "about 50 degrees C" is a maximum temperature; that PTO reading is not a reasonable one. Applicant argues that the Mark patent is a further example of the belief then held by those skilled in this art that lower temperatures were needed for optimum results in direct sulfonation reactions. Mark as co-inventor has supported this view with declarations of record.

Both the Solicitor and the applicant rely on the Gilbert book which, at page 67, discusses the reaction of benzene with sulfur trioxide under various conditions. Gilbert states:

> With both reagents in the vapor phase, a 50% yield of sulfone is obtained at 150-200 degrees C, and 30% at 70-80 degrees C. . . . Addition of SO[3], either as a liquid or vapor, to liquid [**9] benzene gives 15-18% sulfone, but addition of liquid benzene to liquid SO[3] yields 7.5%.

[*1041] Hedges argues that this counters Gilbert's general statement, on which the PTO places great emphasis, that "potentially, the most attractive and practical procedure for sulfonating benzene and other aromatics is by direct reaction with SO[3], since the process is instantaneous, smoothly exothermic, and can involve simple mixing of the two liquids". Hedges points out that despite these "potential" advantages, Gilbert's specific example of the "simple mixing of two liquids" gave only a 15-18% yield.

In contrast to Gilbert's 15-18% yield from the reaction of sulfur trioxide with liquid benzene, Hedges obtained a 96% yield from the reaction of sulfur trioxide with liquid diphenyl sulfone. Other portions of Gilbert, discussed by both the PTO and Hedges, are equally subject to conflicting interpretation. We agree with Hedges that Gilbert cannot fairly be given the predictive virtues attributed to it by the Solicitor.

Hedges argues that he sulfonates liquid diphenyl sulfone at high temperature without the expected charring or reduced yields, and that "the totality of [**10] the prior art disclosures leads substantially away from the claimed invention". We agree with Hedges that [HN4]the prior art as a whole must be considered. The teachings are to be viewed as they would have been viewed by one of ordinary skill. *Kimberly-Clark v. Johnson & Johnson*, 745 F.2d 1437, 1454, 223 U.S.P.Q. (BNA) 603, 614 (Fed. Cir. 1984); *In re Mercier*, 515 F.2d 1161, 1165, 185 U.S.P.Q. (BNA) 774, 778 (CCPA 1975). "It is impermissible within the framework of section 103 to pick and choose from any one reference only so much of it as will support a given position, to the exclusion of other parts necessary to the full appreciation of what such reference fairly suggests to one of ordinary skill in the art". *In re Wesslau*, 353 F.2d at 241, 147 U.S.P.Q. at 393. Hedges correctly points out that the references all suggest that lower temperatures of reaction are preferable. No reference suggests that diphenyl sulfone may advantageously be reacted in the molten state with sulfur trioxide. The data provided by Hedges show significant advantages [**11] of the claimed invention; these data are not challenged by the PTO.

[HN5]On balance, Hedges proceeded contrary to the accepted wisdom. This is "strong evidence of unobviousness". *W. L. Gore & Assoc., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1552, 220 U.S.P.Q. (BNA) 303, 312 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851, 105 S. Ct. 172, 83 L. Ed. 2d 107 (1984), citing *United States v. Adams*, 383 U.S. 39, 15 L. Ed. 2d 572, 86 S. Ct. 708 (1966).

The PTO decision that the invention of claims 8-10 would have been obvious in terms of 35 U.S.C. § 103 is reversed.

REVERSED

RELATED PROCEEDINGS APENDIX

None